Studies on the Phenolic Pungent Principles in Plants and Cultures of *Zingiber officinale* Roscoe

by

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Declaration

I hereby declare that this thesis was composed by myself and the work described herein to be my own, except where indicated otherwise

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The master said:"To hear much, select what is good, and follow it;
to see much and remember it;
these are the steps by which knowledge
[or wisdom] is attained"

Confucius

Abbreviations

aufs	attenuated units full scale
BAP	6-benzylaminopurine
BSA	bovine serum albumin
CPA	p-chlorophenoxyacetic acid
CPM	counts *per* minute
ca.	*circa* (approximately)
cm	centimetre(s)
°C	degree(s) centigrade
µCi	microcurie
mCi	millicurie
2,4-D	2,4-dichlorophenoxyacetic acid
DPM	disintegrations *per* minute
dw	dry weight
ed(s)	editor(s)
e.g.	for example, *exempli gratia*
et al.	*et alii*
Et_2O	diethyl ether
EtOAc	ethyl acetate
EtOH	ethanol
Fig(s)	figure(s)
fw	fresh weight
g	gram(s)
µg	microgram(s)
mg	milligram(s)
h	hour(s)
H#	quench
HPLC	high performance liquid chromatography
ID	internal diameter
i.e.	*id est* (that is)
IOD	integrated optical density
Kin	kinetin
l	litre(s)
µl	microlitre(s)
ml	millilitre(s)
λ	wavelength
LLE	liquid-liquid extraction
LTSEM	low temperature scanning electron microscopy
M	molar
mM	millimolar
mm	millimetre(s)
MeOH	methanol
min	minute(s)
MS	Murashige and Skoog

N	normal
NAA	naphthaleneacetic acid
nm	nanometer(s)
No.	number
OD	optical density
P	probability that the null hypothesis is correct
PAL	phenylalanine ammonia lyase
PCV	packed cell volume
PGRs	plant growth regulators
pH	negative log of the hydrogen ion concentration
phe	phenylalanine
PPPs	phenolic pungent principles
psi	pounds *per* square inch
%	percentage
rpm	revolutions *per* minute
RITA	radioactivity intelligent thin layer analyser
R_f	retention factor
R_T	retention time
SA	specific activity
se	standard error of the mean
sec	second(s)
SH	Schenk and Hildebrandt
TAL	tyrosine ammonia lyase
TCA	trichloroacetic acid
TLC	thin layer chromatography
2D-TLC	two dimensional TLC
tyr	tyrosine
UV	ultra violet
v/v	volume *per* volume
w/v	weight *per* volume
xg	centrifugal force relative to earth's gravitational force

ABSTRACT

The aim of this project was to investigate the phenolic pungent principles [PPPs] in plants and cultures of *Zingiber officinale* Roscoe.

It was confirmed that the PPPs of ginger are present in the underground parts of the plant, chiefly in the rhizome, but these compounds also appear to a much lesser extent in the adventitious roots. The major constituents of this pungent fraction are a homologous series of [6]gingerol (4,8,10,12) and [6]shogaol (4,6,8). These secondary compounds occur together with flavonoid-like compounds and constituents of the essential oil of the spice in the yellow pigmented cells which are also storage locations for oil. It has been shown that a positive correlation exists between the number of these yellow cells and the amount of [6]gingerol.

A range of culture systems were established in order to study the metabolism of these PPPs. Callus cultures, initiated from emerging axillary buds taken from the rhizome, displayed a number of coloured cells initially but the amount of PPPs per g fw declined sharply together with the number of coloured cells per section as the cultures aged. However, when the data were plotted in a per explant basis an increase was observed.

Attempts to influence the production and accumulation of these secondary compounds in differentiating cultures (roots, shoots and plantlets) were partially successful. Generally, in a per g fw basis there was a similar response to that observed during callus induction in which there was a decrease in the amounts of PPPs and also in the number of yellow cells per section. Conversely when the amounts of PPPs were expressed per explant a clear increase was observed which was higher than that obtained during callus induction. Additionally, regenerated plants with mini-rhizomes contained higher amounts of these pungent principles suggesting that higher differentiated structures were necessary for a higher accumulation.

Studies with suspension cultures showed that in some instances the PPPs accumulated after the cessation of growth (20-30d) and were released into the medium; although in other cultures it was shown that these compounds were retained within the cells and appeared during the early stages of growth. Manipulation of the culture medium by the addition of glutamine did not prevent variation in culture pH, and failed to increase the amounts of [6]gingerol and related compounds. Increasing the concentration of sucrose in the culture medium depressed production of the PPPs. Attempts to boost production by the addition of sunflower oil to suspension cultures, to remove the PPPs from the medium, resulted in the appearance of detectable amounts of these compounds within the added sunflower oil. However, overall product yield was not increased. Conjugated forms of [6]gingerol and related compounds were produced in response to the addition of [6]gingerol to suspension cultures.

Radioactive feeding experiments were performed to explore some aspects of the biosynthetic pathway leading to [6]gingerol. Several radioactive putative precursors ([U-^{14}C]*p*-coumaric acid, [U-^{14}C]cinnamic acid, and [^{14}C-methyl]ferulic acid) were fed to pieces of rhizome and to suspension cultures. Some incorporation of radioactivity from [U-^{14}C]cinnamic acid into [6]gingerol was evident with both sources of plant material with greater incorporation of radioactivity into [6]gingerol with suspension cultures.

The implications of these results for the metabolism of the PPPs in plants and cultures of ginger is discussed.

CHAPTER 1

INTRODUCTION

1.1 Historical, taxonomic and morphological aspects of ginger, *Zingiber officinale* Roscoe

The geographical origin of ginger is uncertain since there are no records of the plant being found in the wild, although it appears to be of Indian origin and then spread to China and other areas of Asia. (Ridley, 1912). Ginger has been used as a spice and medicine since ancient times, especially in India and China (Purseglove, 1975). This spice seems to have been one of the earliest known to the Europeans and was certainly used by the Greeks and Romans who obtained it from Arab traders who brought it from India. The Portuguese took it to West Africa and other parts of the tropics in the 16th century. As the rhizomes are easily transported in a living state the plant was rapidly introduced into the New World soon after its discovery. It was introduced to New Spain (Mexico) by Francisco de Mendoza and from there to Jamaica (one of the main producers) and there are records of ginger being exported from Jamaica to Spain in 1547 (Ridley, 1912).

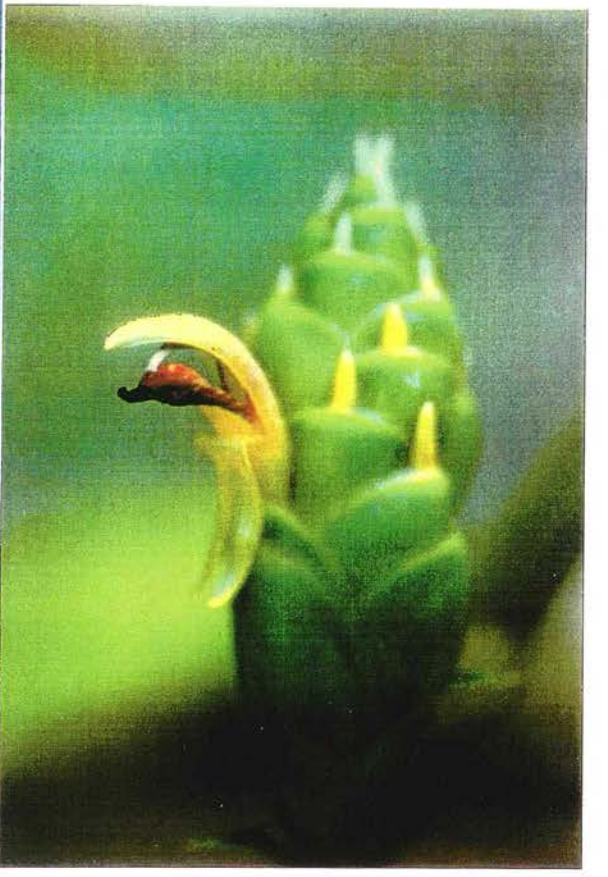
Ginger, *Z.officinale* Rosc is a monocotyledonous herbaceous perennial plant belonging to the family Zingiberaceae in the order Zingiberales, a family of *ca.* 47 genera and 1400 species comprising several important spice crops, including *Afromomum* (grain of paradise), *Amomum* , *Curcuma* (Turmeric), *Elettaria* (cardamom), and *Zingiber* (ginger).

The genus *Zingiber* contains about 80-90 species of perennial rhizomatous herbs with ginger being the most important of this genus. Ginger possesses a white yellowish pungent aromatic rhizome covered with scale leaves, which gives the importance of the spice and produces at intervals leafy shoots about 30-100cm tall and 5mm in diameter. The aerial pseudo-stems usually bear 8-12 leaves with thin lamina, which are subsessile, linear-lanceolate, darkish green in colour and usually 5-20cm long and 1-3cm wide (see Fig. 1.1a). The inflorescence is rarely formed (15-20cm long) and it arises directly from the rhizome usually in a separate pseudo-stem (see Fig. 1.1b). The inflorescence is initially sealed with bracts and the flowers are

produced in the axil of each bract and are fragile and short-lived. The flowers are usually sterile and the plant only occasionally sets seed and is generally propagated by division of the rhizome (Purseglove, 1975).

Figure 1.1 (a) ginger plant about 2 months old grown under greenhouse conditions (see 2.1), **(b)** inflorescence of a ginger plant grown under greenhouse conditions approximately 7 months after planting

(a) **(b)**



1.2 Chemical constituents of *Z.officinale*

The constituents of ginger can be separated into two major groups, which appear in different fractions following extraction, the oleoresin and the volatile or essential oils.

1.2.1 Oleoresin

The oleoresin or non-volatile oil of ginger contains the pungent principles of the spice representing 4.5 to 7.5% of the dry weight (Govindarajan, 1982a-b). There are also some flavouring components, which are chiefly found in the essential oil and are present together with fatty acids, resins, carbohydrates and colouring matter.

The components of the oleoresin can be extracted with several organic solvents such as acetone, methanol, diethyl ether and dichloromethane. A number of studies have been conducted investigating the kinetics of extraction using different organic solvents. These studies report that acetone is the best of those solvents tested (Kandiah and Spiro, 1990; Spiro and Kandiah, 1989, 1990, Spiro *et al.*, 1990). Therefore the extraction method employed routinely in this investigation for the extraction of the phenolic pungent principles [PPPs] from different plant tissues was performed using acetone employing a modified version of that described by Spiro and Kandiah (1989).

The different pungent compounds present in the ginger oleoresin are listed in Table 1.1 and the chemical structures of the three main compounds are presented in Fig. 1.2.

Table 1.1 Pungent principles found in ginger oleoresin

[n]gingerol	n=1-4, 6, 8, 10, 12	[n]gingerdione
[n]shogaol	n=4, 6, 8, 10	hexahydrocurcumin
zingerone		[n]paradol
dehydrogingerol		gingeryl diacetate
gingerdiol		methylgingerol

The gingerols, the main natural pungent principles of ginger, consist of a series of aldols each containing a phenolic group. The homologous series n=4, 6, 8 predominate and the others are minor constituents. [n]Shogaol and zingerone have also been detected although it has been suggested that these compounds appear as artefacts after extraction or long term storage (Middleditch *et al.*, 1989). The proportion of gingerol to shogaol and zingerone is considered to be a quality parameter, thus fresh good quality oleoresin contains a large amount of gingerol with a very low amount of shogaol. The higher the proportion of shogaol the lower the pungency (Connell, 1969) and it is generally accepted that pungency decreases in the order gingerol>shogaol>zingerone and that the [6] homologs are more pungent than the others. However, some quite recent reports claim that [6]shogaol has a higher pungency than [6]gingerol (Govindarajan, 1982ab and Fisher, 1992). This would explain the high pungency exhibited by stored oleoresin which may exceed that of freshly extracted oleoresin although the latter contains more aroma constituents which are mainly present in the essential oil fraction.

1.2.2 Volatile or essential oil

The essential oil provides the flavour and fragrance of the spice and represents approximately 1.5-2.5% of the dry weight (Govindarajan, 1982ab). This fraction is obtained by steam distillation of the rhizome (Bednarczyk and Kramer, 1975; Smith

and Robinson, 1981 and Ibrahim and Zakaria, 1987). Other methods of extraction employing liquid CO_2 have been reported and these seem to provide a higher yield and more stable compounds (Gopalan and Manahdhar, 1991). This distillate however, does not contain any of the pungent principles and is made up of about 4% monoterpenes, 65% sesquiterpenes, 17% sesquiterpene alcohols together with small quantities of some aldehydes, ketones and esters. The main constituents of the essential oil are listed in Table 1.2.

Table 1.2 Main volatile components found in essential oil of ginger

Sesquiterpenes	Monoterpenes
ϖ-seliene	carene
β-elemene	α, β-pinene
β-zingiberene	limonene
β-bisabolene	geranial
ar-curcumene	neral
β-farnesene	β-phellandrene
	linalool
	α-terpineol
	sabinene

1.2.3 Chemical relationships between the main pungent principles of *Z.officinale*

Gingerol appears to be the main natural pungent principle in ginger and the compounds shogaol and zingerone seem to be present in lower amounts in the plant and are formed by the action of alkalis, acids or heat during extraction. Two degradation pathways have been described for the production of shogaol and zingerone from gingerol (Connel and McLachlan, 1972 and MacHale *et al.*, 1989). A dehydration reaction via hot acid treatment leads to the formation of shogaol which

by hydrogenation forms paradol, another minor pungent principle present in the grain of paradise (*Amomun melegueta* Roscoe). Zingerone and an aliphatic aldehyde are produced via a retroaldol condensation in the presence of hot alkali (Connel and McLachlan, 1972 and MacHale *et al.*, 1989) (see Fig. 1.2).

1.3 Biosynthetic pathway proposed for [6]gingerol

A biosynthetic pathway for [6]gingerol has been proposed by Denniff *et al.* (1980) in which phenylalanine, coumaric acid, and ferulic acid are precursors, although the two latter intermediates appear to be more effective since they were incorporated more efficiently into [6]gingerol.

Phenylalanine is elaborated to ferulic acid through *p*-coumaric acid; ferulic acid then condenses with malonate and hexanoate by two possible paths:

Path a: ferulate reacts with malonyl CoA to form a β-ketoester (R=SCoA) followed by hydrolysis to R=OH and condensation with hexanoyl CoA.

Path b: hexanoate first reacts with malonyl CoA to yield (R=SCoA) and the product of hydrolysis (R=OH) then reacts with feruloyl CoA.

Both paths (a, b) lead to [6]dehydrogingerdione as the first intermediate possessing the complete C-skeleton of [6]gingerol; then two reaction steps are necessary to transform [6]dehydrogingerdione to [6]gingerol so that either [6]gingerdione or [6]dehydrogingerol must be an intermediate (see Fig. 1.3).

Figure 1.2 Chemical relationships between the main pungent principles of ginger

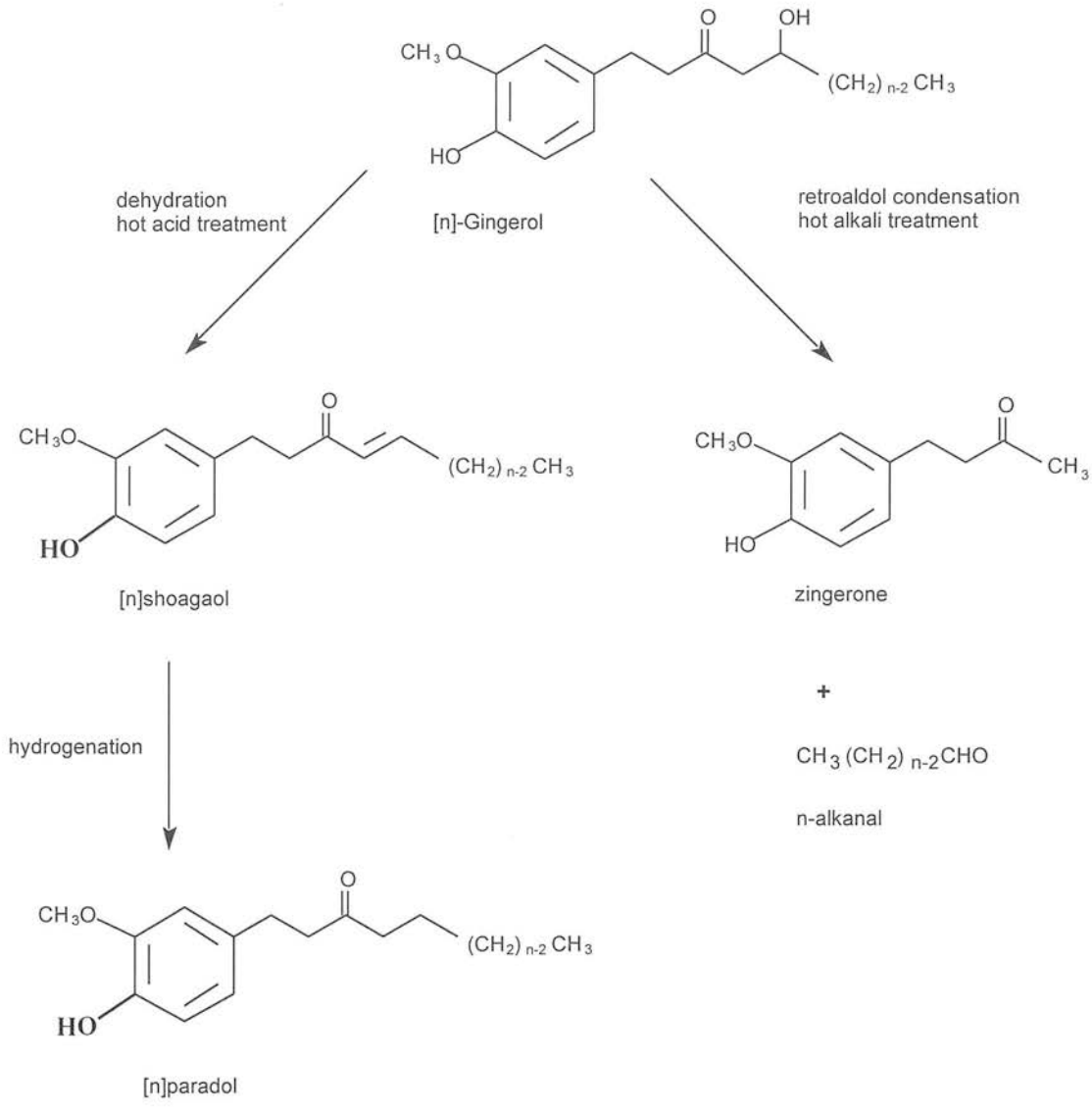
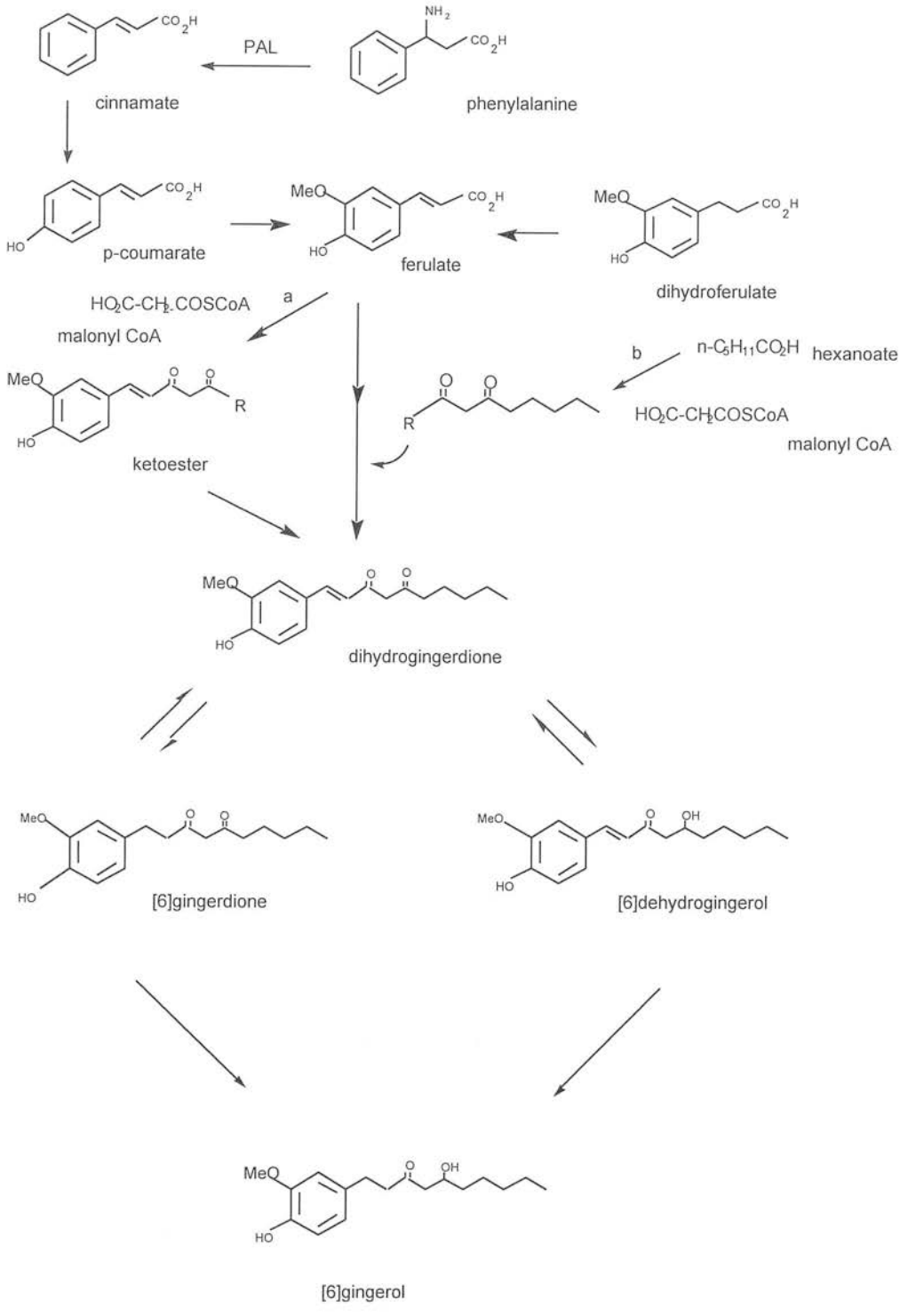


Figure 1.3 Biosynthetic pathway for [6]gingerol proposed by Denniff *et al.* (1980)



1.4 Principal uses of *Z.officinale*

The application of ginger as a food additive to enhance flavour, particularly in Chinese and Indian cuisine, is well known. It is also used in the preparation of ginger bread, and ginger ale. In addition the plant is used extensively in traditional medicine and recently the possible application of ginger in modern medicine has been investigated to provide an effective substitute for commonly dispensed drugs which have disadvantageous side effects. In Table 1.3 several medicinal properties of ginger are listed.

Table 1.3 Medicinal properties of *Z.officinale*

Properties	Reference
Prostaglandin inhibitor	Fumiyuki *et al.* (1982)
Cardiotonic action	Shoji *et al.*(1982); Kobayashi *et al.* (1988)
Anti-oxidant	Lee *et al.* (1986)
Anti-ulcer effect	Yamahara *et al.* (1988)
Against seasickness	Mowrey and Clayson, (1982); Grøntved *et al.* (1988); Holtman *et al.* (1989);
Anti-inflammatory	Elias & Rao (1988)
Anti-rheumatic disorders	Srivastava & Mustafa (1989)
Relief of migraine	Mustafa & Srivastava (1990)
Anti-emetic after surgery	Bone *et al.* (1990); Phillips *et al.* (1993)
Relief of catarrh	Fulder (1993)
Carminative	Fulder (1993)

1.5 Secondary metabolites in plants

Secondary compounds may be defined as natural products, usually of plant origin, which do not function directly in primary metabolic activities such as supporting

growth, development and reproduction of the organism (Conn, 1981) but they do have specific functions in the survival and ecological success of the plant. For example, the colour and scent of flowers may attract insects and/or birds facilitating cross-pollination; other secondary compounds such as phytoalexins (Bell, 1981; Barz *et al.*, 1990) are part of the plant defence mechanisms activated in response to stress or injury; moreover, other secondary substances present in the plant act as deterrents to potential predators (Harborne, 1990). Similarly, secondary products provide an enormous source of useful compounds for mankind, such as gums, food flavourings, pigments, fragrances and pharmaceuticals which are extensively exploited by man. Indeed, it has been estimated that 80% of people living in developing countries are almost completely dependent on traditional medicine practices for their primary health care and higher plants are the main source of drug therapy (Farnsworth, 1990). The importance of secondary metabolites is well known and a large body of research has been conducted to understand their synthesis and to improve product yield mainly with plants. However, recent research has moved toward the production of useful secondary plant products by plant tissue cultures.

1.6 Production of secondary products by plant tissue cultures

Higher plants are able to synthesise a large range of complex compounds useful to man and are generally more efficient at accumulating these substances than cultured plant cells (Fowler, 1983, 1986). However, plant tissue cultures provide an alternative way to produce useful secondary compounds. Many secondary substances can be extracted from plants but a regular supply is uncertain, as many of these valuable plants grow in tropical countries in which political and economical stability is very fragile jeopardising the world supply (Collin, 1987). Although some compounds originally obtained from plants have now been chemically synthesized many secondary substances are chiral molecules and cannot be synthesized easily or cheaply (Wink, 1990). This means that the biological production of these compounds

can compete; moreover, natural products used by the food industry are required to comply with strict legislation. Plant cell cultures have also been used for the biotransformation of specific compounds. For example, the conversion of the cardiac glycoside β-methyl digitoxin to β-methyl digoxin by cell cultures of *Digitalis lanata* (Alfermann *et al.*, 1983). Although the large-scale production of secondary metabolites by plant cell cultures has achieved much attention there are only a few compounds which can be produced at a higher yield than the plant (Fowler, *et al.* 1990). Two well known examples are the production of shikonin by cell suspension cultures of *Lithospermum erythrorhizon* (Fujita *et al.*, 1981 and 1988a), and the production of berberine by cultures of *Coptis japonica* (Fujita, 1988b). However, low product yield is still the major barrier to the commercial production of other useful secondary metabolites by plant cells *in vitro* (Yeoman *et al.*, 1990).

Accumulation of secondary compounds by plant cell cultures often occurs when growth ceases or slows down (Yeoman *et al.*, 1980, Lindsey and Yeoman, 1985) and there is an inverse relationship between growth and product accumulation which corresponds to a switch from primary to secondary metabolism with diversion of common precursors away from biomass production to secondary metabolism (Phillips and Henshaw, 1977). Thus, organized cultures, which usually grow more slowly, would be expected to have limited primary metabolism and accumulate higher yields of secondary metabolites. There are however, examples where the accumulation of secondary compounds takes place during active growth and there is a positive correlation between growth and product yield (Kadkade, 1982 and Sakuta *et al.* 1986; Shaib, 1992). It has also been shown that some secondary compounds accumulate during the lag phase of growth, a period after subculture when there is no growth or cell division and the cells are adapting to a new environment (Miyasaka *et al.*, 1985; Boyd, 1991) but this may be a continuation of production from the previous stationary phase (Noguchi and Sankawa, 1982).

1.7 Attempts to increase the yield of secondary compounds in tissue cultures

There are still a number of obstacles which have to be overcome before plant cell cultures in general can be used for large scale production of valuable secondary compounds. As already mentioned, low product yield is the main barrier for the commercial exploitation of this technique, despite extensive research effort, however, there are several strategies which could lead to increased product yield.

1) cell line selection

2) manipulation of culture conditions

3) induction of differentiation

4) genetic manipulation

1.7.1 Cell line selection

Cultured plant cells exhibit a large variation and are generally unstable in culture as the result of a relatively 'hostile' environment (Yeoman and Forche, 1980). Variation between cultured cells often leads to a deterioration in the ability of these cells to synthesise and accumulate the desired secondary metabolite. There are a number of instances in the literature where a decline or absence in product yield with time has been reported (Deus-Neumann and Zenk, 1984; Morris *et al.*, 1989 and Sierra *et al.*, 1992). It has also been observed that within a population of cells some compounds are highly expressed and high producing cell lines are formed spontaneously, while other pathways are difficult to express and the levels of secondary compounds are very low. However, selected high-yielding clones also show instability leading to a reduction in the amount of product (Deus-Neumann and Zenk, 1984) and re-selection is then necessary to restore product yield. Contrarily, there are a number of examples where cell clones selected for the production of a specific secondary metabolite show a clear stability over a long period of time with

stable secondary product yield and where routine screening was not required (Hall and Yeoman, 1987; Yamada and Hashimoto, 1990).

1.7.2 Manipulation of culture conditions

Manipulation of culture conditions has been successful in stimulating biosynthetic pathways leading to the accumulation of the desired secondary compound. Therefore having established a high yielding cell line several culture parameters can be altered to enhance product yield, these include: alteration of the concentration of minerals such as nitrogen or phosphorus (Mantell and Smith, 1983 and Lindsey, 1985), varying the concentration of the carbon source (Do and Cormier, 1991; Roper *et al.*, 1985); and changing the concentration and proportion of plant growth regulators (Payne *et al.*, 1991 and Shaib, 1992). Variation in pH, temperature and the light regime has also been reported to affect product yield (Mantell and Smith, 1983). In addition the application of precursors to a particular metabolic pathway has resulted in an increase in product accumulation (Berlin and Witte, 1982; Lindsey and Yeoman, 1984a). Exposing cells to elicitors has also been shown to boost the production of secondary metabolites in many cultures (Eilert *et al.*, 1985; Funk *et al.*, 1987; Messner and Ball, 1993).

It is generally accepted that slow growing cultures which show some degree of cell aggregation appear to accumulate higher yields of secondary compounds. This has encouraged the development of several systems for cell immobilization employing an inert matrix. Immobilization reduces growth rate and may induce partially organised structures which produce higher yields than freely suspended cells (Lindsey *et al.*, 1983; Lindsey and Yeoman, 1984b, and Lindsey, 1986a).

1.7.3 Induction of differentiation

It has been mentioned previously that it is generally accepted that slow growing cultures show higher product accumulation (Yeoman *et al.*, 1982) and this appears to coincide with cell aggregation which enhances cell differentiation as already explained. Immobilization methods have been used to increase product yield by reducing growth rate and encouraging differentiation by promoting close cell-cell contact (Lindsey and Yeoman, 1986; Yeoman, 1987a). Cell-cell contact encourages the formation of aggregates which display a much greater degree of cell differentiation than freely suspended cells. There are also often signs of organisation into definite structures such as roots, shoots, proembryoids and embryoids (Yeoman, 1987) which could increase secondary metabolite yield. Brodelius (1984) also reported that immobilization prolongs the stationary phase of growth, where secondary metabolite production has been shown to take place (Yeoman *et al.*, 1980), thus stimulating the production of secondary substances. Several substrata have been employed to immobilize cultured cells and to enhance product yield (see Table 1.4); however, some of the substrata used affect cell viability due to their toxicity, for example the use of polyacrylamide, but some success has been achieved and higher product yields have been reported after cell immobilisation (Lindsey and Yeoman, 1986; Kaetsu *et al.*, 1990).

Table 1.4 Some examples of substrata used for cell immobilization of plant cells

Substratum	Culture	Reference
Polyacrylamide	*Umbilicaria pustulata*	Mosbach & Mosbach (1986)
Calcium alginate	*Digitalis lanata*	Alfermann *et al.* (1980)
Reticulate polyurethane	*Capsicum frutescens*	Lindsey *et al.* (1983), Mavituna & Park (1985)

Another approach towards increasing secondary metabolite yield is to induce organogenesis in order to restore the metabolic characteristics of the original explant. The major means of inducing regeneration is to change the composition of the nutrient medium usually by an alteration to the amounts or the ratio of PGRs mainly auxin and cytokinin. For example the regeneration of leafy shoots of *Chrysanthemum cinerariaefolium* stimulated the accumulation of pyrethrins (Zieg *et al.*, 1983), and the induction of shoot proliferation in cultures of *Lavandula latifolia* increased the accumulation of monoterpenes (Calvo and Sánchez-Gras, 1993); also a sharp rise in alkaloids content was obtained when cultures of a *Duboisia* sp were induced to form roots (Endo and Yamada, 1985), similarly the production of emetine and cephaeline was enhanced in root cultures of *Cephaelis ipecacuanha* (Jha *et al.*, 1991)

Another approach involves the use of 'hairy root' cultures which are permanently differentiated structures. This has been successful in raising the amounts of secondary metabolites especially in those cases where roots are the main site of synthesis. Hairy roots are formed when explants are successfully infected with *Agrobacterium rhizogenes* and undergo various metabolic changes caused by the insertion of a small fragment of bacterial DNA (Ri-DNA plasmid) (Chilton *et al.*, 1982). This stimulates rapid root growth through altering the metabolism of endogenous PGRs (Stafford, 1991).

Established 'hairy root' cultures proliferate indefinitely in simple media without the addition of PGRs. Most importantly these roots can accumulate the desired secondary substances through many sub-cultures and often the yields achieved are much higher than those found in the intact plant. For example, Maldonado-Mendoza *et al.* (1993) reported yields of the alkaloids hyoscyamine and scopolamine two fold higher than the plants of *Datura stramonium* from which these 'hairy root' cultures had been established. Likewise, Ogasawara *et al.* (1993) obtained spectacular and sustained production of naphthoquinone, 50 times that of the mother plant by a 'hairy root' culture of *Sesamum indicum*. Also using this culture system, two new anthraquinones were also produced.

1.7.4 Genetic manipulation

The different strategies for increasing product yield already described have been successful. However, little is known or understood about the genetic control of secondary metabolism and how it is regulated. In particular, it is very difficult to establish whether a key enzyme controls a particular pathway or as is generally believed a group of coordinated enzymes which participate in the complex control of metabolism. Thus targeting a particular enzyme for cloning and genetic manipulation, although very tempting may not achieve the desired effects.

Recent advances in molecular biology and the recombinant DNA technologies have enabled a genetic approach to elucidate the complexities of secondary metabolism which could alter metabolism and boost product yield. Most of the published reports have been concerned with the phenylpropanoid pathway, for example Liang *et al.*, (1989), Dixon and Lamb (1990) and Loake *et al.*, (1991, 1992) although there are some reports on alkaloid synthesis (Songstad *et al.*, 1991) and tannin biosynthesis (Carron *et al.* 1994).

These examples highlight the potential of these techniques for the manipulation of regulatory pathways. Possibly in the near future plant biotechnologists will be able to target specific enzymes involved in a certain pathway, to clone a particular gene controlling the expression of regulatory gene(s) and perhaps remove the major barrier to low product yield in plant cell cultures.

1.8 Aims and objectives

The aim of this investigation was to study the phenolic pungent principles [PPPs] in plants and cultures of *Z.officinale* Roscoe. The following objectives were attempted :

1) To determine the nature, distribution and cellular localization of PPPs in the plant.

2) To establish callus, suspension and regenerating cultures to enable metabolic studies on the PPPs.

3) To improve product yield by modifying the culture medium of suspension cultures.

4) To discover the effectiveness of various putative precursors to the biosynthetic pathway leading to [6]gingerol employing both suspension cultures and explants of ginger rhizome.

CHAPTER 2

MATERIALS AND METHODS

2.1 Plant material

The species *Zingiber officinale* Roscoe of the family Zingiberaceae was used throughout this investigation.

Plant stocks were established from *Z.officinale* rhizomes by vegetative propagation since the plant rarely flowers and sets seed.

The rhizomes, which were Brazilian in origin, were purchased in the local supermarket. It was not possible to identify the variety of ginger employed, but it is likely to be Rio de Janeiro, commonly cultivated in and exported from Brazil.

Pieces of rhizome *ca.* 5-8cm in length with buds were planted individually 3 to 5cm below the soil surface (see Table 2.1) in pots 25cm in diameter and 25cm in depth, in a greenhouse within a plastic chamber heated electrically by tubular heaters. This provided a temperature range of 22-28°C for the soil and 17-33°C for the chamber. A light regime of 16h was supplied using 400w bulbs (Mercury Ballast Fluorescent Reflector Upright General Electric Co). The pots were watered regularly to maintain a moist soil. Under these conditions axillary buds began to elongate after 10-15d (see Fig. 2.1)

Initially, different types of soil were used in order to establish the most suitable medium to support rapid growth of the pieces of rhizome. Table 2.1 shows the types of soil used and the growth response of the rhizome pieces after 10-15d.

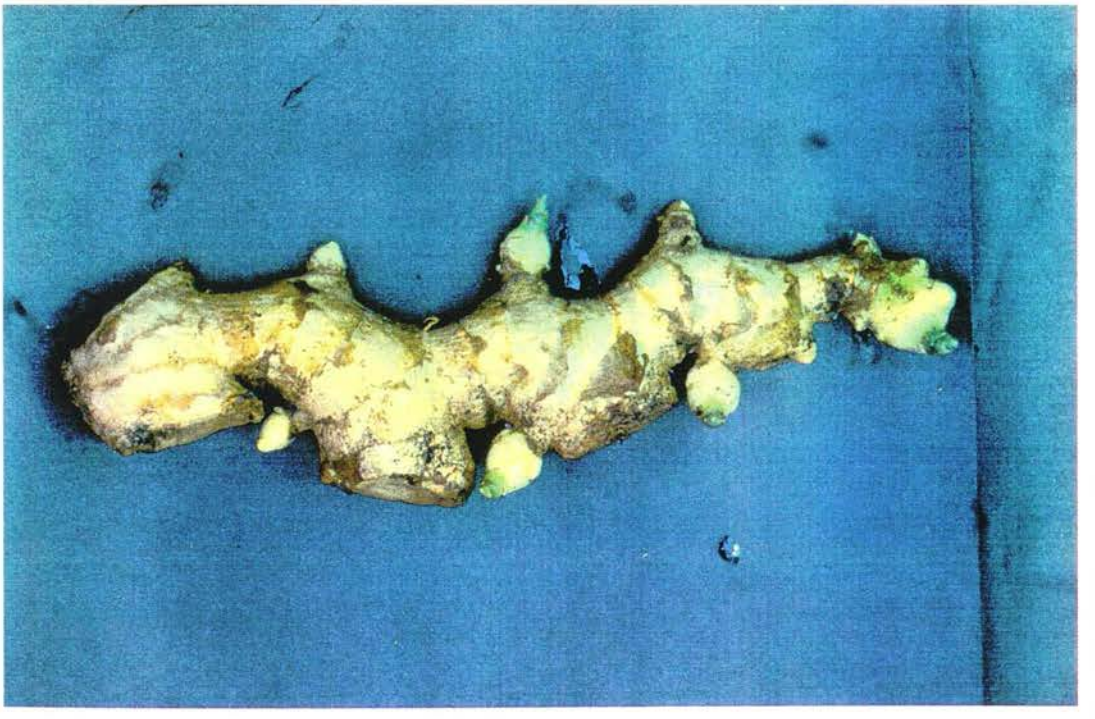
Table 2.1 Types of soil employed for the cultivation of *Z.officinale* rhizomes.

Type of soil	Response
John Innes compost No.2	Fast
Mixture of Levington and fine sharp sand (1:1)	Slow
Mixture of Levington and Perlite (1:1)	Satisfactory
Mixture of Levington, Perlite and Vermiculite (1:1:1)	Satisfactory
Pure sharp sand	Very slow

It can be seen from Table 2.1 that John Innes compost No.2 was the best for the development of buds and therefore was used routinely.

Elongation of axillary buds was followed by the appearance of adventitious roots and the emergence of shoots from the soil. These emerging buds, 10-15d, were used for the initiation of *Z.officinale* cultures.

Figure 2.1 Appearance of a *Z.officinale* rhizome after 10-15d cultivated under the conditions stated above. Observe the development of the axillary buds.



2.2 Tissue and cell culture

2.2.1 Preparation of culture media and growth regulator stock solutions

2.2.1.1 Preparation of culture media

Cultures of *Z.officinale* were initiated and maintained on two main basal media, Murashige and Skoog (MS) (1962) and Schenk and Hildebrandt (SH) (1972), supplied by Imperial laboratories, UK. To these media, were added 3% sucrose (Fisons, Loughborough, UK) and various combinations of plant growth regulators 2,4-D, BAP, CPA, NAA as well as Kin (see 2.2.1.2, 2.2.2.3, 2.2.2.4, 2.2.2.5) (all obtained from Sigma Chemical Co., Dorset, UK). The pH of the nutrient medium was adjusted to 5.8 with N HCL or N KOH before the addition of agar and before autoclaving. For the preparation of solidified culture medium, 0.8% (w/v) bacterial agar No. 1 (Oxoid, Unipath Ltd., Hampshire, UK) was used.

2.2.1.2 Growth regulator stock solutions

Stock solutions of the following growth regulators were prepared

2,4-D (2,4-dichlorophenoxyacetic acid)

BAP (6-benzylaminopurine)

NAA (naphtaleneacetic acid)

CPA (*p*-chlorophenoxyacetic acid)

Kin (6-furfurylaminopurine)

Preparation of plant growth regulator stock solutions was carried out by dissolving 100 mg of each PGR in an appropriate solvent which was then made up to 100ml with distilled water. Initially, 2,4-D and CPA were dissolved in 5ml of EtOH. BAP, NAA and Kin were dissolved in 5ml of 0.1N KOH. These stock solutions were stored at 4°C in the refrigerator and renewed every 2-3 months.

2.2.2 Sterilisation and aseptic work

2.2.2.1 Sterilisation by heat

Sterilisation of culture media, distilled water, glassware and instruments was carried out by autoclaving at 121°C for 20min. at a steam pressure of 15 psi.

2.2.2.2 Sterilisation with sodium hypochlorite

Ten to fifteen day old emerging buds of the *Z.officinale* rhizome were employed as a source of material for culture initiation. Surface sterilisation was undertaken by removing the scale leaves of the excised emerging buds using a pair of forceps and a scalpel. The buds were then washed under running tap water for 5-10min., followed by a brief immersion in 80% (v/v) EtOH for 30sec, and then immersed in a 20% (v/v) solution of hydrogen peroxide (from a 30% stock solution, Sigma) for 4min. The buds were then transferred to a 20% (v/v) solution of sodium hypochlorite (1.5% available chlorine) with 4-5 drops of a wetting agent (Tween-80) which was stirred by means of a magnetic stirrer for 30-35min. Finally, the buds were rinsed five times with sterile distilled water to remove the residual sodium hypochlorite.

2.2.2.3 Sterilisation with mercuric chloride

The surface sterilisation procedure was carried out as with sodium hypochlorite except that a 1% (w/v) solution of mercuric chloride was used and the material was immersed for 15min, then washed five times with sterile distilled water.

2.2.2.4 Sterilisation with ethanol and aseptic work

All aseptic cell culture manipulations were carried out on the bench of a laminar air flow cabinet in a stream of sterile air. Before use, absolute ethanol was sprayed on

the surfaces of the laminar air flow cabinets then wiped off with a paper towel. Instruments were kept in absolute ethanol and flamed immediately before use.

2.2.3 Initiation and maintenance of cultures

2.2.3.1 Plant inocula

After surface sterilisation, the emerged axillary buds (see 2.2.2.2), were cut transversely into slices *ca.* 3mm in thickness, and 2-3 cylinders were removed from each slice with a cylindrical cork borer 4mm in diameter. Each cylindrical inoculum 3x4mm, with a fresh weight of approximately 25-30mg, was placed aseptically on the surface of 10 to 12ml of solidified culture medium contained in a 5.0cm diameter sterile polystyrene Petri dish (Sterilin Ltd., Hounslow, UK) which was then double sealed with Parafilm 'M' (American National Can Co., Greenwich, USA) to avoid desiccation of the medium and to prevent contamination. The Petri dishes were then placed in an environmentally controlled culture suite at 25±2°C, with a light intensity of 25μmol.$m^{-2}sec^{-1}$ photon flux density, illuminance 1050 lux (Compton Warmwhite fluorescent tubes).

2.2.3.2 Callus culture

Callus was induced by inoculating explants on different culture media with the following composition:

1/2 M+S	S+H
3% sucrose	3% sucrose
1mg/l 2,4-D	1mg/l 2,4-D
0.5mg/l BAP	0.1mgl^{-1} Kin
0.8% agar No.1	1mgl^{-1} CPA
	0.8% agar No.1
pH 5.8	pH 5.8

The culture medium (10-12ml) was contained in small polystyrene Petri dishes of 5.0cm in diameter. Inocula were placed onto the culture medium as described in 2.2.3.1. The slow growing callus cultures were subcultured every 6 weeks by dividing the callus into pieces of *ca.* 0.5cm and transferring, under aseptic conditions, on to Petri dishes containing fresh medium.

2.2.3.3 Plant regeneration medium

Plant regeneration was achieved by culturing explants on two different culture media with the following composition:

MS	MS
3% sucrose	3% sucrose
5mg/l BAP	3mg/l BAP
1mg/l NAA	0.5mg/l NAA
0.8% agar No.1	0.8% agar No.1
pH 5.8	pH 5.8

The Petri dishes containing the inocula were treated as described in 2.2.3.1.

2.2.3.4 Suspension culture

The same culture media as those employed for the establishment of callus cultures were used for the initiation of suspension cultures but without the addition of agar (see 2.2.3.2).

Initially, *ca.* 0.5g of the most friable pieces of callus were transferred aseptically into small Erlenmeyer flasks of 125ml capacity containing 20ml of sterilised liquid medium. The flasks were then sealed with a double layer of aluminium foil and placed on an orbital shaker at 98 rpm and 8mm amplitude, at a light intensity of

25μmol.$m^{-2}sec^{-1}$ photon flux density, illuminance 1050 lux (Compton Warmwhite fluorescent tubes) and a temperature of 25±2°C.

The growing cultures were subcultured into fresh medium every 4-5 weeks. When the biomass was large enough the suspended cells were transferred into larger Erlenmeyer flasks of 250ml capacity with 40ml of liquid medium. The cultures were filtered through a sterilised nylon mesh (pore size 64μm) (Lockertek, Warrington, UK) and, using a perforated spoon spatula, *ca.* 0.5-0.75g wet weight of cell was transferred to fresh liquid growth medium.

2.2.4 Characterisation of culture growth

2.2.4.1 Determination of fresh weight

Fresh weight (fw) of callus was determined after carefully removing any agar adhering to the culture, the callus was then placed in a paper cup and weighed on a digital balance (Sartorius 1412 MP8, Sartorius Instruments Ltd., Surrey, UK).

The fresh weight of a cell suspension culture was determined after the cells had been removed from the liquid medium by filtration through a Whatman No.1 filter paper (Whatman lab sales Ltd., Kent, UK). The separated cells were then transferred to a paper cup, weighed on a Sartorius digital balance and the fw recorded.

2.2.4.2 Determination of dry weight

Dry weight (dw) was measured after the callus or suspended cells had been left in a pre-heated oven at 70°C for 36h. The dried material was cooled in a desiccator and weighed on a digital balance.

2.2.4.3 Determination of packed cell volume

Suspended cells grown in 250ml Erlenmeyer flasks containing 40ml of liquid medium were poured into 50ml calibrated polystyrene centrifuge tubes (J.Bibby Science Products, Staffordshire, UK) and centrifuged at 300xg for 10min in a bench centrifuge (IEC Centra 4R Centrifuge, Intl. equipment Co. Bedfordshire, UK). The size of the cell pellet was recorded and the packed cell volume (PCV) calculated as follows:

$$PCV = \frac{\text{vol. of packed cell}}{\text{total volume}} \times 100$$

2.2.4.4 Determination of the proportion of suspended pigmented cells

Using a Pasteur pipette 0.50ml of a cell suspension was placed on a grid microscope slide (S-7 England finder, Graticules Ltd., Kent, UK). Excess liquid was then removed and a few drops of a 10% (w/v) solution of sodium carbonate, known to intensify the pigment colour, were added. A cover slip was then placed on top of the preparation and the cells counted using a monocular compound microscope (Vickers Instruments Ltd.) at 200x magnification.

Cell counting of both pigmented and non-pigmented cells enclosed in the circles of the grid slide finder (Graticules Ltd.) was performed and the percentage of pigmented cell (PPC) calculated as follows:

$$PPC = \frac{\text{No. pigmented cells}}{\text{Total No. of cell counted}} \times 100$$

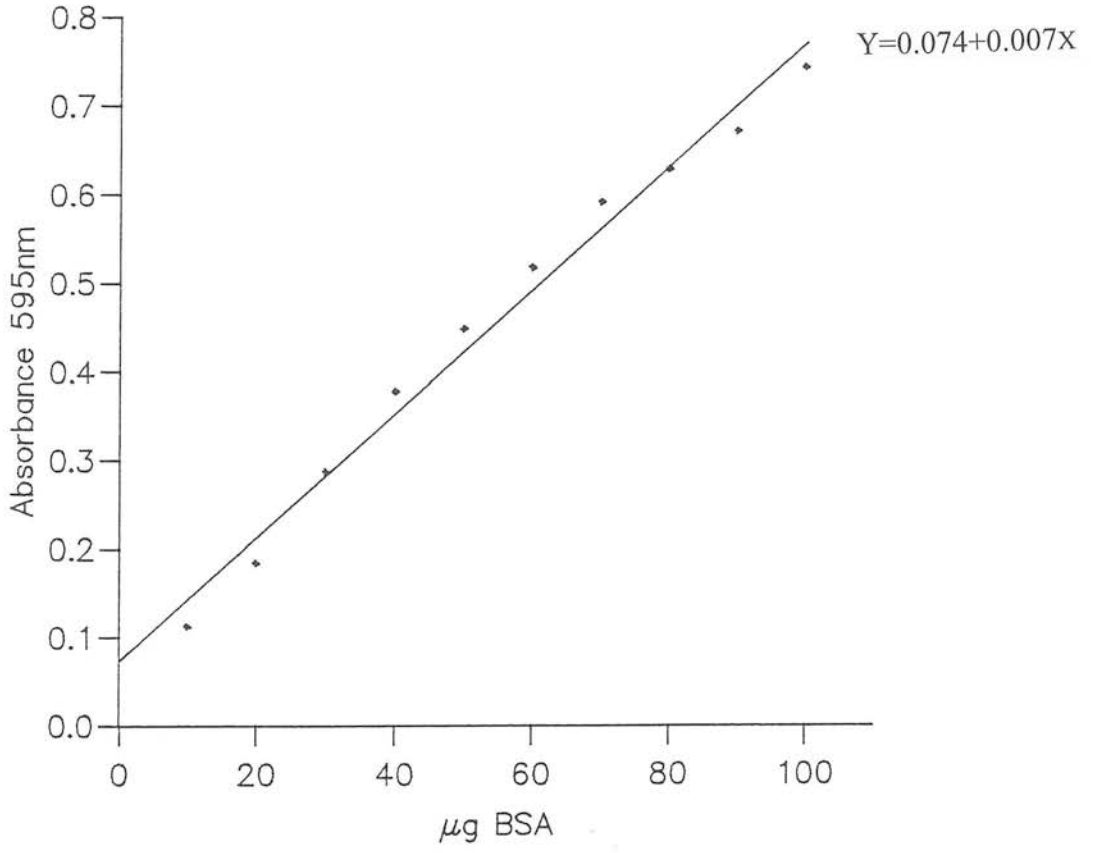
2.2.4.5 Determination of protein content

Protein content was determined using the tissue residue remaining after extraction of the phenolic compounds (see 2.3.2), following the method of Bradford (1976). The assay volume was altered to 5.2ml.

The protein reagent was prepared by dissolving 50mg Coomasie Brilliant Blue G-250 (Sigma) in 25ml absolute ethanol. The solution was then homogenised using a Sorvall Omnimixer 4x for 2min and diluted to 400ml with distilled water and stirred overnight in the dark at room temperature. After the addition of 50ml of concentrated ortho-phosphoric acid (BDH) the reagent was adjusted to 500ml, vacuum filtered through a Whatman No.1 filter paper (Whatman), and stored at room temperature in the dark.

Extraction of protein was carried out by immersion of the tissue (0.1-0.3g) in 0.1N NaOH (8ml) for 2h. The mixture was then centrifuged at 2000xg at 4°C for 10min, the supernatant transferred, and the pellet re-extracted with a further 8ml of 0.1N NaOH for another 2h, then centrifuged as described above and the supernatant taken up and pooled with the initial supernatant fraction. One ml of the combined supernatants was taken and the extracted protein precipitated by adding trichloroacetic acid (TCA) (Fisons) to give a final concentration of 15% (v/v) and placed on ice for 2h. The mixture was then centrifuged in an Eppendorf centrifuge at *ca.* 12000xg for 10min. The supernatant was discarded and the protein pellet re-dissolved in 1ml of 0.1N NaOH. Samples were diluted as required and aliquots of 200µl were thoroughly mixed together with 5ml of the protein reagent. After 5min, the absorbance of the solution was measured at a wavelength of 595nm using a Philips PU 8625 VIS/UV spectrophotometer against a blank consisting of 5ml of reagent and 200µl 0.1N NaOH. Protein concentration was estimated using BSA (bovine serum albumin, Sigma) to construct a calibration curve (see Fig. 2.2)

Figure 2.2 Calibration curve for the determination of protein as described in 2.2.4.5. The plotted line is the regression line (see 2.11) with a correlation coefficient of 0.932.



2.3 Extraction and chemical analysis of the phenolic pungent principles of *Z.officinale*

2.3.1 Extraction of phenolics from *Z.officinale* rhizome

Ginger rhizomes were surface cleaned with running tap water, dried with a paper towel and cut with a scalpel into slices of approximately 1-2mm in thickness. The slices were placed in a freeze-dryer under vacuum and freeze dried for 3d at -57°C. The dried material was then powdered in a food processor. Approximately 5g of the dried ground ginger powder was then placed in 75ml of acetone (Analytical grade, (Fisons) and extracted by stirring for 4h. The extract was then filtered under vacuum through a Whatman No.1 filter paper and evaporated to dryness using a rotary evaporator over a warm water bath at 35°C. The extract was then taken up in 5ml MeOH (HPLC grade, BDH Ltd, Poole, UK) filtered through a 0.45μm pore Nylon membrane filter (Whatman) held in a Millipore filter unit (Millipore, UK Ltd, Watford) attached to a syringe. The solution was expelled with the syringe piston and collected in a vial sealed with a crimp top (Phase Separation, Clwyd, UK) and stored at -20°C until required for analysis.

2.3.2 Extraction of phenolics from cultures

2.3.2.1 Callus cultures and regenerated plants

Phenolics were also extracted from established *Z.officinale* callus cultures and regenerated plants (see 2.2.3.2 and 2.2.3.3). After the fresh weight had been recorded (2.2.4.1), the material was cut into small pieces and then ground up in a pestle and mortar together with some 40-100 mesh sand (BDH) and a small volume of acetone (*ca.* 4ml). Once the plant material had been ground up, an extra 30-40ml of acetone was added to the mortar and the slurry transferred to a 100ml Erlenmeyer flask, the mixture was then extracted by stirring at room temperature for 4h. The extract was then filtered as described in 2.3.1, evaporated to dryness under vacuum on a warm

water bath at 35°C, then taken up in 1ml HPLC MeOH (BDH) and filtered through a 0.45µm Nylon filter as described in 2.3.1. Finally, the samples were concentrated to a volume of 250µl under a stream of nitrogen and the samples stored in a 2ml autosampler vial sealed with a crimp top at -20°C until required for analysis.

2.3.2.2 Suspension cultures

Suspended cells grown and maintained as described in 2.2.3.4 were separated from the liquid medium by filtration under vacuum through a Whatman No.1 filter paper, the fresh weight recorded (see 2.2.4.1) and the cells ground up and extracted as described in 2.3.2.1. The extract was then taken up in 1ml MeOH (HPLC grade, BDH Ltd, Poole, UK) and filtered through a 0.45µm pore Nylon membrane filter (Whatman) held in a Millipore filter unit (Millipore, UK Ltd, Watford) attached to a syringe. The solution was expelled with the syringe piston and collected in a 2ml vial sealed with a crimp top (Phase Separation, Clwyd, UK) and stored at -20°C until required for analysis. When required the volume of sample was reduced to 250µl under a stream of nitrogen prior to HPLC analysis

2.3.2.2.1 Extraction of the liquid culture medium

Liquid medium from the suspension cultures was also extracted for phenolic compounds. After removal of the suspended cells, the liquid medium was subjected to liquid liquid extraction (LLE) with an equal volume of the solvent mixture 1:1 (v/v) diethyl ether, ethylacetate (Fisons). LLE of the liquid medium was carried out 3x, and separation of the aqueous and organic phase was achieved using a separating funnel. The water in the organic phase was removed with 3g of anhydrous sodium sulphate (Sigma) filtered and evaporated to dryness under vacuum on a warm water bath at 35°C. Finally, the residue was taken up in 1ml of HPLC MeOH (BDH) then filtered through a Nylon filter as described in 2.3.2.1 and 2.3.2.2, and concentrated

down to 250µl under a stream of nitrogen. Samples were stored in a 2ml autosampler vial sealed with a crimp top at -20°C until required for further analysis. The aqueous fraction of the LLE was retained for analysis and was then hydrolysed both with acid and alkali (see 2.8.1, 2.8.2).

2.4 Extraction of flavonoids from flowers of four different species

Flower petals of four different species; *Rosa canina*; *Senecio cineraria*; *S. jacobaea* and *Verbascum blattaria*, were employed for the extraction of flavonoids. The petals (13g) were homogenised in 100ml methanol (Analytical grade, BDH) using an electric blender at maximum speed 4x for 1min. at 20sec. intervals. The homogenate was then stirred overnight in 500ml methanol at room temperature. The combined extract was filtered through a Whatman No.1 filter paper and evaporated to dryness using a vacuum rotary evaporator over a warm water bath at 35°C. The residue was then dissolved in 100ml of water and partitioned 3x with equal volumes of chloroform (Analytical grade, BDH). The aqueous fraction was again partitioned 3x with equal volumes of ethyl acetate (Analytical grade, BDH). The final three fractions were evaporated to dryness as described above and taken up in 3ml of water, whilst the remaining two fractions (chloroform, ethylacetate) were taken up in 3ml 80% (v/v) methanol-water and stored in the freezer at -20°C. Spectrophotometric analysis of flavonoid were carried out for comparison with compounds present within the yellow-pigmented cells present in ginger rhizome and roots

2.5 Analysis of phenolic pungent principles by thin layer chromatography (TLC)

2.5.1 Absorbents and solvent systems

Thin layer chromatography was performed using 20x20cm silica gel 60 plastic plates, with or without fluorescent indicator, with a thickness of 200μm (E. Merck, Darmstadt, Germany).

For preparative scale separation, TLC was carried out using 20x20cm K5 silica gel glass plates with a thickness of 250μm (Whatman).

Several solvent systems were used throughout this investigation. For the separation of phenolic compounds from a crude ginger extract Solvent System I, a variation of the mixture used by Bhagya (1977), consisting of a mixture of toluene-methanol (80:5) was used routinely.

In order to separate the general phenylpropanoids such as ferulic acid, coumaric acid, and cinnamic acid which run together with the ginger phenolics, a second solvent system, Solvent System II, a mixture of toluene-acetic acid (90:20) was employed.

Two dimensional TLC (2D-TLC) was also performed. Solvent System II was employed for the first direction and Solvent System III, a mixture of chloroform-ethylacetate-acetic acid (50:50:1), for the second direction.

Chromatography tanks (Alltech) were equilibrated with the appropriate solvent system before elution of the plates. Samples were loaded onto the plates 1.5cm apart using a Hamilton microsyringe with the volume of the sample varied (10-50μl) depending upon the expected concentration of phenolics. Standards were loaded and run together to assist in the identification of phenolics. Plates were eluted in sealed tanks until the solvent front was *ca.* 2cm from the top of the plates. They were

removed from the tank and left in a fume cupboard to remove the solvent before further processing.

2.5.2 Visualisation of separated phenolics on TLC plates

2.5.2.1 Short/Long wave ultraviolet light

Separated compounds on TLC plates with a fluorescent indicator can be easily visualised under short wavelength (254nm) ultraviolet light (UV) in a dark room.

When TLC plates without a fluorescent indicator were used some of the phenolics fluoresced under long wavelength (366nm) ultraviolet light (UV).

2.5.2.2 Folin-Ciocalteu

This reagent was supplied by BDH. After development, the plate was allowed to dry and then sprayed with this reagent using a Humbrol spray gun at a distance of approximately 25cm. After 40-60sec phenolics on the plate gave grey blue spots. This reagent was used initially when a crude extract of ginger was analysed. When TLC plates with ginger extract were run together with other phenolics, especially phenylpropanoids such as ferulic acid, cinnamic acid, and coumaric acid, the reagent mixture 1% potassium ferrycianide with 0.5% ferric chloride was used (see 2.5.2.3).

2.5.2.3 Ferric chloride-potassium ferricyanide

This reagent was freshly made up prior to spraying by mixing equal volumes of 1% (w/v) ferric chloride and 0.5% (w/v) potassium ferricyanide in water. The plates after being dried, were sprayed with this reagent as described in 2.5.2.2. Phenolics give a blue to reddish colour.

2.5.2.4 Iodine fumes

Fumes of iodine can be used as a general visualising agent for compounds with a double bond. With most of the ginger phenolics this reaction is reversible and the colour produced after exposure of the plate to the iodine fume disappears within minutes. Visualisation of the compounds was achieved after exposing the plate in a sealed TLC tank saturated with iodine fumes. Those compounds with a double bond appear yellow-brownish in colour.

2.6 Analysis of phenolic pungent principles by high performance liquid chromatography (HPLC)

High performance liquid chromatography (HPLC) was used routinely for the separation and quantification of phenolics in extracts.

2.6.1 High performance liquid chromatography system

HPLC was performed using a Gilson 302 liquid chromatograph fitted with a C18 Lichrosorb reverse-phase column 200x4.6 mm ID (Capital HPLC Specialists, Bathgate, Scotland, UK) and a Gilson Holochrome UV/VIS detector. The 20µl sample was injected into the system using a Gilson autosampling injector 231-401 which was controlled by an IBM PS2 model microcomputer using the Gilson HPLC system controller 714 V1.2. All data processing was performed using the same software.

2.6.2 Preparation of HPLC mobile phases

The two solvents of the mobile phase, methanol and water, were of HPLC grade (BDH). To remove any particulate matter present in the solvents, MeOH was filtered under reduced pressure through a 0.45µm pore Nylon-66 membrane filter (Rainin

Instruments Co). Water was also filtered through a 0.45μm pore cellulose acetate membrane (Schleicher and Schull, Surrey, UK). Prior to use, filtered solvents were de-gassed for 15-20min by passing a stream of helium (BOC) through them to prevent any air bubbles from entering the HPLC system.

2.6.3 Preparation of samples for HPLC analysis

Samples were extracted as described in 2.3.1, 2.3.2, 2.3.2.2 and 2.3.2.2.1. Samples for HPLC were taken up in 1ml MeOH HPLC grade. These solutions were then filtered through a 0.45μm pore Nylon membrane filter (Whatman) held in a Millipore filter unit (Millipore, UK Ltd, Watford) attached to a syringe. The solution was expelled with the syringe piston and collected in a 2ml vial with a crimp top (Phase Separation, Clwyd, UK) and stored at -20°C until required for analysis. When necessary after filtration the volume of the samples was reduced to 250μl under a stream of nitrogen (BOC). Samples were then ready to be injected and analysed on HPLC.

2.6.4 Operating conditions for HPLC analysis

The mobile phase used was a variation of the method described by Chen *et al.* (1986), and consisted of a gradient 65% up to 80% MeOH-water then 100% MeOH followed by a re-equilibration to 65% prior to the next injection (55min gradient). The flow rate was 1ml min^{-1} and detection was performed at 282nm, with UV sensitivity set at 0.2 aufs. Prior to each run, external standards of [6]gingerol, [8]shogaol and [6]shogaol, kindly donated by Dr. Koroyanagi (Shizuoka University, Japan), were injected onto the column at a range of concentrations to enable the system software to calculate the amount of phenolic in the samples from the area under each peak. The running time for each sample was 55min and the retention

times (R_T) displayed by the standard were 8.70min for [6]gingerol, 16.45min for [4]shogaol and 23.25min for [6]shogaol.

Figure 2.3 shows an HPLC chromatogram of a mixture of [6]gingerol, [6]shogaol and [4]shogaol when injected and analysed under the conditions described in 2.6.4 and Figure 2.4 shows three calibration curves of the standards used with the appropriate regression lines fitted.

Figure 2.3 HPLC chromatogram of a mixture of [6]gingerol, [6]shogaol and [4]shogaol injected and analysed under the conditions described in 2.6.4.

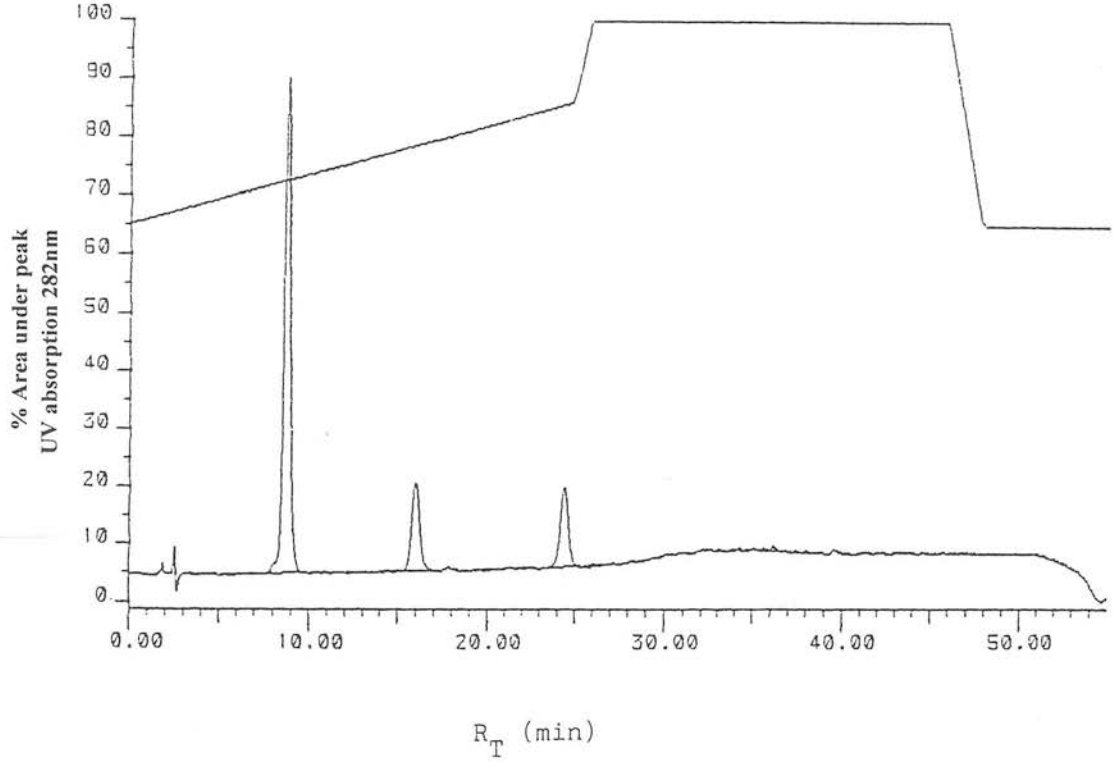
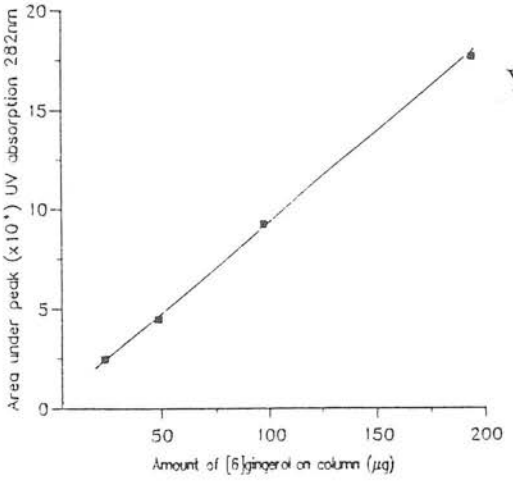
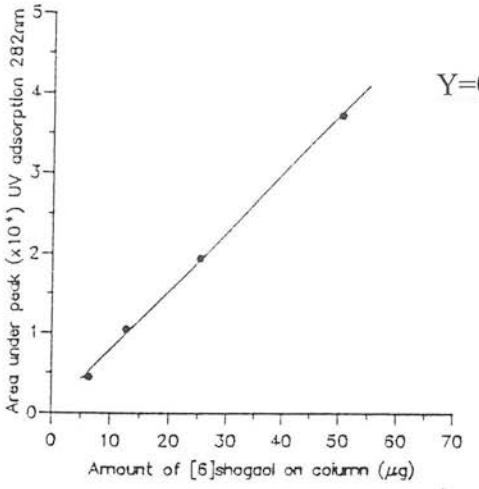


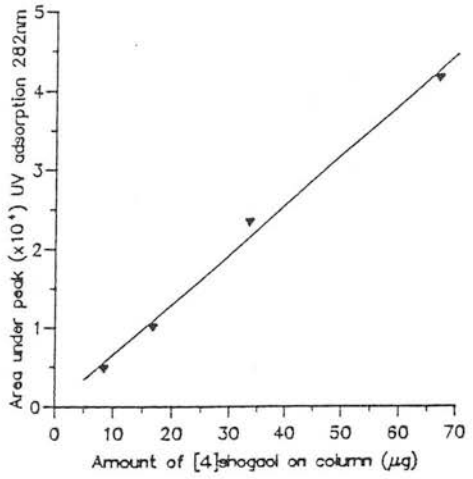
Figure 2.4 Peak areas obtained for [6]gingerol, [6]shogaol and [4]shogaol analysed by HPLC using the conditions described in 2.6.4. The plotted lines are the regression lines (see 2.11). The correlation coefficients were for [6]gingerol 0.99, for [6]shogaol 0.997, and for [4]shogaol 0.989.



Y=0.149+0.092X



Y=0.055+0.073X



Y=0.025+0.063X

2.7 Microscopic studies

2.7.1 Light microscopy of sections from *Z.officinale* rhizome

Cylindrical pieces of ginger rhizome from the cortex and medulla, of *ca.* 4-5mm in diameter and 45-50mg in fw were taken, mounted onto a specimen stub 12.5mm in diameter (Agar Scientific Ltd., Essex, UK) with a cryo-adhesive, (Tissue-Tek Agar Scientific Ltd.), and frozen immediately by immersion in liquid nitrogen for 3-4min. The stubs were then placed in a cryostat set up at -17°C. After temperature equilibration, 30 sections of 25-30μm in thickness and 4-5mm in diameter were removed from the top, middle and lower parts of the tissue cylinder (10 sections from each region) and mounted on a microscope slide in a drop of water. Once the sections had thawed they were ready for observation using a Vickers monocular compound microscope (Vickers Instruments Ltd., UK) at a magnification of x200. The number of yellow cells present in the 30 cryo-sections was counted.

2.7.2 Low temperature scanning electron microscopy of *Z.officinale* rhizome sections

Low temperature scanning electron microscopy (LTSEM) was performed using a Cambridge Stereoscan S250 microscope (Cambridge Scientific Instruments Co., Cambridge, UK) and an EMscope cryo-preparation system SP2000 (Biorad Microscience Division, UK).

Hand sections from a mature ginger rhizome 1-2mm in thickness were mounted with cryo-adhesive (Tissue-Tek) on stubs with a series of surface grooves (Jeffree and Read, 1991). The specimens were then frozen by immersion in slushy pre-cooled nitrogen under a dry argon atmosphere at -210°C for 5-7 min, followed by fracture in a preparation chamber under vacuum. The frozen material was then etched by sublimation of the water under controlled conditions at -70°C for 10-15 min. Finally, the etched specimens were gold coated and examined under LTSEM.

2.7.3 Microspectrophotometric measurements

Microdensitometry is a technique which allows precise measurements of light absorption by cell chromophores in microscopic preparations (Cowden, 1973; Chapria and Ellis , 1984).

Microspectophotometric analysis of the cell contents of individual yellow cells of the cryo-sectioned plant material was performed using a Vickers M85 scanning integrated microdensitometer (Vickers Instruments Ltd., York, England). Preparation of the sections for these measurements was as described in 2.7.1. An absorption spectrum was obtained for individual cells by measuring the integrated optical density (IOD) over a wavelength range of 400-700nm at 10nm intervals.

2.7.4 Preparation of different histochemical reagents

Several histochemical reagents were prepared for microscopic studies of cryo-sections of ginger in attempts to identify the range of compounds present within the yellow pigmented cells

It has been reported (Mangalakumari *et al.*, 1984) that a 10% (w/v) aqueous solution of sodium carbonate when applied to tissue sections produces a change in colour of the content of some ginger cells from yellow to red. This prepared solution was used for histochemical studies.

In order to detect phenolic compounds a mixture of 1% (w/v) ferric chloride and 0.5% (w/v) potassium ferricyanide in water was employed (BDH). Phenolics give a blue colour with this reagent (Stahl, 1965). Finally, for the localisation of lipidic material Nile red (Greenspan *et al.*, 1985)(Sigma Chemical Co. Ltd., Poole, U.K.) was used. A $1mgml^{-1}$ solution of Nile Red in 10ml of acetone (Analytical grade, Fisons, U.K.) was prepared and from this concentrated solution, 10µl was taken and dissolved in 2ml of water; this final solution was used for staining the sections which

were examined by fluorescence microscopy using green light excitation (Olympus 1M, G filter), which produces a red fluorescence with lipids.

2.8 Alkaline and acid hydrolysis of *Z.officinale* extracts

2.8.1 Alkaline hydrolysis

In order to investigate the possibility of glycosylation of the phenolics in suspension cultures after LLE (see 2.3.2.2.1), the aqueous fractions were subjected to alkaline hydrolysis, which breaks the ester bonds of the conjugate. Prior to that, the aqueous fraction was reduced to a volume of *ca.* 5-10ml under vacuum on a warm water bath at 40°C and any excess of organic solvent used during LLE eliminated. Alkaline hydrolysis was performed with N KOH giving a pH of 10.5, the mixture was left overnight at room temperature to allow hydrolysis, followed by LLE 3x with equal volume of the solvent mixture 1:1 (v/v) Et_2O-EtOAc. The combined extract was evaporated to dryness as described in 2.3.2.2.1 and the samples prepared and analysed on TLC and/or HPLC analysis (see 2.5)

2.8.2 Acid hydrolysis

Acid hydrolysis breaks the phenolics conjugated with sugars via O-glycosylic bonds, this reaction also cleaves ester bonds.

After alkaline hydrolysis (see 2.8.1), the samples were acid hydrolysed under the following experimental conditions: aqueous fractions with a volume of *ca.* 5-10ml were mixed with 1.5ml 6N HCl giving a pH of 0.4. The samples were then heated in a hot water bath set up at *ca.* 80-90°C for 2h then allowed to cool down and extracted 3x with equal volume of the solvent mixture 1:1 (v/v) Et_2O-EtOAc as described in 2.3.2.2.1 and the samples prepared and analysed on TLC and/or HPLC analysis (see 2.5).

2.9 Initial steps for isolation of *Z.officinale* pungent principles using two rapid techniques of column chromatography

2.9.1 Flash chromatography

A glass chromatography column 135cm in length with an ID of 2cm supplied by Aldrich (Gillingham, UK) was employed. As described by Still *et al.* (1978) to prepare the column, 9.5g of silica gel 60 (40-60μm, Merck 9385) was added slowly giving an effective column length of 15cm. An 0.8cm layer of sand 40-100 mesh (BDH) was placed on the top of the dry gel bed. Then 40ml of the solvent system I, a mixture of toluene-MeOH (80:5) (see 2.5.1) was added to the column and pressure applied from a nitrogen cylinder to remove the solvent from the column, remove any trapped air and to produce an even packed column; an extra 200ml of this solvent mixture was then passed through the column to ensure an even compact column. To the column 2ml of extracted sample from freeze-dried ginger rhizome powder (see 2.3.1) dissolved in solvent system I was applied. After the whole sample had been adsorbed by the column, 200ml of solvent system I was added, and pressure applied to produce a flow rate of 50mm per min (read from the decrease in the level of the solvent above the column bed) and 20 fractions of a volume of 10ml collected. The compounds present in each fraction were analysed by TLC employing the solvent system I and visualisation of the developed plate was achieved after spraying with Folin-Ciocalteu (see 2.5.1; 2.5.2.2).

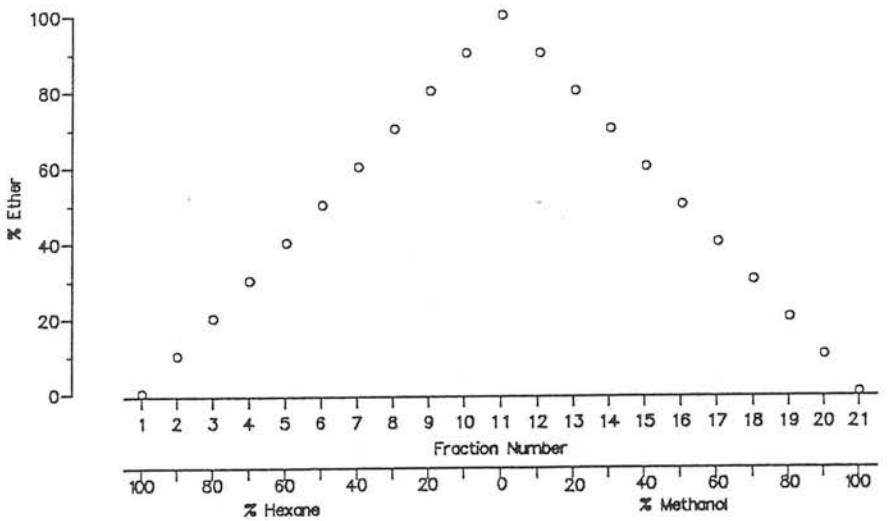
2.9.2 Vacuum chromatography

A Büchner flask (100ml) fitted with a sintered filter funnel with an ID of 3.5cm was connected to a water pump. To the funnel, 9.5g of silica gel 60 (Merck) was added to produce an evenly packed silica column of 3cm in length.

A 3ml sample extracted from freeze-dried ginger rhizome powder was mixed with 2g of silica gel 60 and the solvent removed using a rotary evaporator over a warm

water bath at 35°C. The dried silica containing the sample was spread evenly over the top of the silica column. A piece of Whatman No. 1 filter paper (Whatman) with the same diameter as the ID of the funnel was placed on top of the column to prevent damage during addition of the solvent. Hexane (25ml) was added slowly, and the solvent allowed to penetrate the whole column, then the solvent was sucked out with a water pump to facilitate rapid elution and the eluent was collected as the first fraction. Further elution was performed using 20 aliquots of 25ml of solvent mixtures with increasing polarity, composed of hexane-diethylether and diethylether-methanol, as shown in Fig. 2.5. The different fractions were collected in test tubes and the volume reduced to 10ml under a stream of nitrogen and then analysed by TLC as described above.

Figure 2.5 Composition of the solvent system used in vacuum chromatography (21 different aliquots).

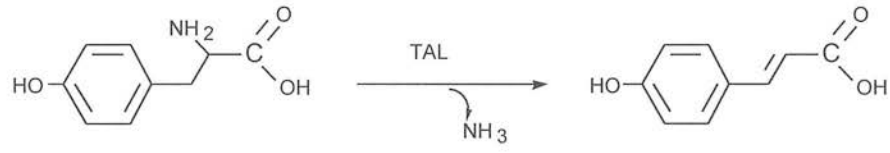


2.10 Radiolabelling and measurement of radioactivity

2.10.1 Synthesis of radioactive putative intermediates of [6]gingerol

2.10.1.1 Synthesis of [U-^{14}C]*p*-coumaric acid

p-Coumaric acid has been described as a precursor in the biosynthesis of [6]-gingerol (Denniff *et al.*, 1980). [U-^{14}C]*p*-coumaric acid was synthesized from L-[U-^{14}C] tyrosine (Amersham) with an SA of 468 mCi/mmol by a deamination reaction employing an enzyme preparation of PAL (phenylalanine ammonia lyase) from *Rhodotorula glucinis* grade II (Sigma) enzyme which converts phenylalanine into cinnamic acid but also possesses a lower TAL (tyrosine ammonia lyase) activity which converts tyrosine to *p*-coumaric acid (see diagram).



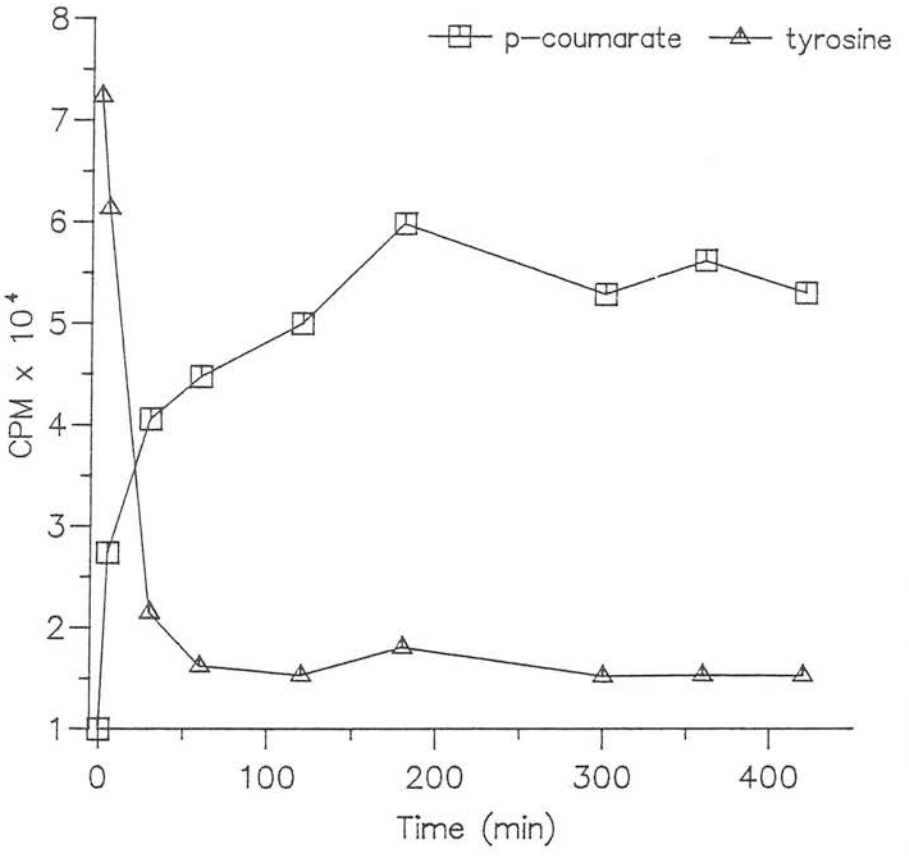
tyrosine coumaric acid

A preliminary experiment was carried out to study the rate of conversion of L-tyrosine to *p*-coumaric acid by incubating a mixture of 4µg of L-tyrosine (200µl), 15µl of PAL enzyme, corresponding to 0.08 units, in 50µl of 50mM Tris buffer pH 8.5 at 30°C. In addition to this, 5µCi of L-[3-5-^{3}H]tyrosine (5µl) was added to the mixture to facilitate measurement of the conversion, 10µl samples of the reaction mixture were removed after 5min, 1h, 3h, 6h, and 9h. The samples were loaded onto a silica gel 60 TLC plate and run using solvent system II. Tyrosine does not migrate using this solvent system and remains at the origin. The TLC plate was first autoradiographed to localise the radioactive compounds present (see 2.10.3.1) then the radioactive spots were scraped from the TLC plate, mixed with 5ml of scintillation fluid and counted (2.10.3.3). It can be seen from Fig. 2.6 that after 1h

most of the tyrosine has been converted to *p*-coumaric acid. Separation of the reaction product was achieved by preparative TLC (see 2.5.1) but a loss of product was observed following this procedure. It was therefore necessary to devise a different extraction method as well as other reaction conditions.

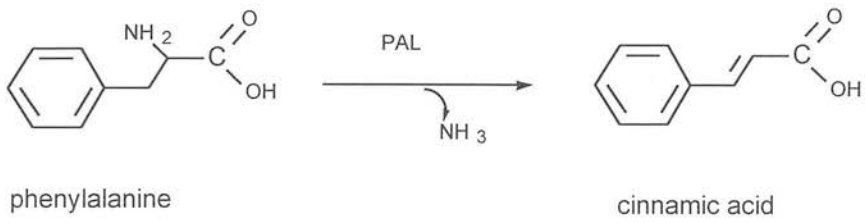
A modification of the conditions of the preliminary experiment was made to perform the final conversion of L-[U-^{14}C]tyrosine to [U-^{14}C]*p*-coumaric acid using PAL enzyme. L-[U-^{14}C]tyrosine (22.5μCi) in an aqueous solution containing 2% EtOH, was dried down using a speed-vac at room temperature, then re-dissolved in 0.5ml of distilled water. To this 125μl of 50mM Tris buffer pH 8.5 was added together with 0.25 units of PAL from *Rhodotorula glucinis* grade II (Sigma), corresponding to a volume of 57.25μl of the enzyme preparation dissolved in 60% glycerol, 3mM Tris-HCL pH 7.5. The mixture was incubated on a shaker at 200rpm at 30°C for 16h. After that, 0.1ml of 6N HCl was added to stop the reaction and to ionise the aminoacid L-[U-^{14}C]tyrosine, which after being ionised would remain in the aqueous fraction. The [U-^{14}C]*p*-coumaric acid formed was extracted 5x with 1.5ml of EtOAc (Fisons). The combined extracts were dried down employing a rotary evaporator over a warm water bath at 30°C and the residue was taken up in 1ml MeOH HPLC grade then filtered through a 0.45μm nylon filter and stored in the refrigerator at 5°C until required. The purity of the product was checked using TLC. The radioactivity of the [U-^{14}C]*p*-coumaric acid formed and the L-[U-^{14}C]tyrosine remaining was measured (2.10.3.3) and showed a conversion of 87.10%.

Figure 2.6 Time course of the conversion of tyrosine to *p*-coumaric acid. [3,5-^{3}H]tyrosine was used to follow the reaction under the conditions stated in 2.10.1.1.



2.10.1.2 Synthesis of [U-^{14}C]cinnamic acid

The method was similar to that used for the synthesis of [U-^{14}C]*p*-coumaric acid from L-[U-^{14}C]tyrosine (2.10.1.1). [U-^{14}C]cinnamic acid was synthesized from [U-^{14}C]phenylalanine (see diagram) with an SA of 450mCi/mmol (Amersham) using PAL enzyme from *Rhodotorula glucinis* grade II (Sigma).



phenylalanine cinnamic acid

The reaction was performed using 20µCi of [U-^{14}C]phenylalanine which was dried down from an aqueous solution containing 2% EtOH using a vac-speed at room temperature, then re-dissolved in 0.5ml distilled water. To this, 125µl of 50mM Tris buffer pH 8.5 was added together with 0.25 units of PAL enzyme which corresponded to a volume of 57.25µl of the enzyme preparation dissolved in 60% glycerol, 3mM Tris-HCl pH 7.5. The mixture was incubated on a shaker at 200rpm at 30°C. After a period of 16h the reaction was stopped by adding 0.1ml 6N HCl which ionises the phenylalanine so that it remains in the aqueous fraction. The [U-^{14}C]cinnamic acid formed was extracted 5x with 1.5ml of EtOAc. The combined extracts were dried down in a rotary evaporator over a warm water bath at 30°C. The residue was taken up in 1ml of MeOH HPLC grade then filtered through a 0.45µm nylon filter and stored in the refrigerator at 5°C until required. The radioactivity of the [U-^{14}C]cinnamic acid formed and the [U-^{14}C]phenylalanine remaining was measured (2.10.3.3) and showed a conversion of 87.0%.

2.10.2 Radiolabelling

Three different radioactive compounds, putative precursors of the [6]-gingerol biosynthetic pathway as described by Denniff *et al.* (1980) were employed for feeding experiments. These three different radioactive precursors were [U-^{14}C]*p*-coumaric acid, [U-^{14}C]cinnamic acid and [^{14}C-methyl] labelled ferulic acid, kindly donated by Dr. K. Myton (Edinburgh University).

Radioactive feeding experiments were performed using these compounds employing two different types of material. Initially 20ml of 35d old suspension cultures (2.2.3.4) were fed with [U-^{14}C]*p*-coumaric acid. Another group of experiments was performed employing pieces of growing ginger rhizomes 3 months old with aerial parts. The rhizome was surface sterilised as previously described in 2.2.2.2 and cylindrical pieces of *ca.* 1g fw taken by means of a cork borer of 4mm in diameter. Sterilised pieces were then placed in Petri dishes of 5cm in diameter on top of moist filter paper. The radioactive compounds were applied by dripping the MeOH solution from a micro-syringe onto the tissue slowly to ensure absorption of the solution by the tissue. The amount of radioactivity added was 0.3μCi of [U-^{14}C]*p*-coumaric acid and [U-^{14}C]cinnamic acid, and 0.5μCi of [^{14}C-methyl] labelled ferulic acid.

2.10.3 Measurement of radioactivity

2.10.3.1 TLC-autoradiography

Samples from the radioactive feeding experiments described in 2.10.2 were loaded onto TLC plates and developed using solvent system II (2.5.1).

Autoradiography was employed for the localisation of radioactive spots on TLC plates. Autoradiographs were obtained by contacting Hyperfilm-MP (Amersham, UK) to a radioactive chromatogram placed in a 21.5x25cm autoradiographic cassette

in a dark room under an X-ray safelight (Ilford 914, Ilford, UK). The cassette was then closed and sealed with autoclave tape, placed in a black plastic bag and exposed for 3 to 4 weeks at -20°C depending upon the expected radioactivity of the samples loaded. Films were left at room temperature prior to development using an X-OGRAPH compact X-2 automatic film processor (X-OGRAPH Ltd, Malmesbury, Wilts, UK).

2.10.3.2 TLC-RITA

RITA (Radioactivity Intelligent Thin Layer Analyser) was employed for rapid measurement of the distribution of radioactivity on TLC plates. RITA scans were carried out on an Isomass IM-3000 Radio TLC Analyzer (Nuclear-Interface, Münster, Germany).

2.10.3.3 Liquid scintillation counting

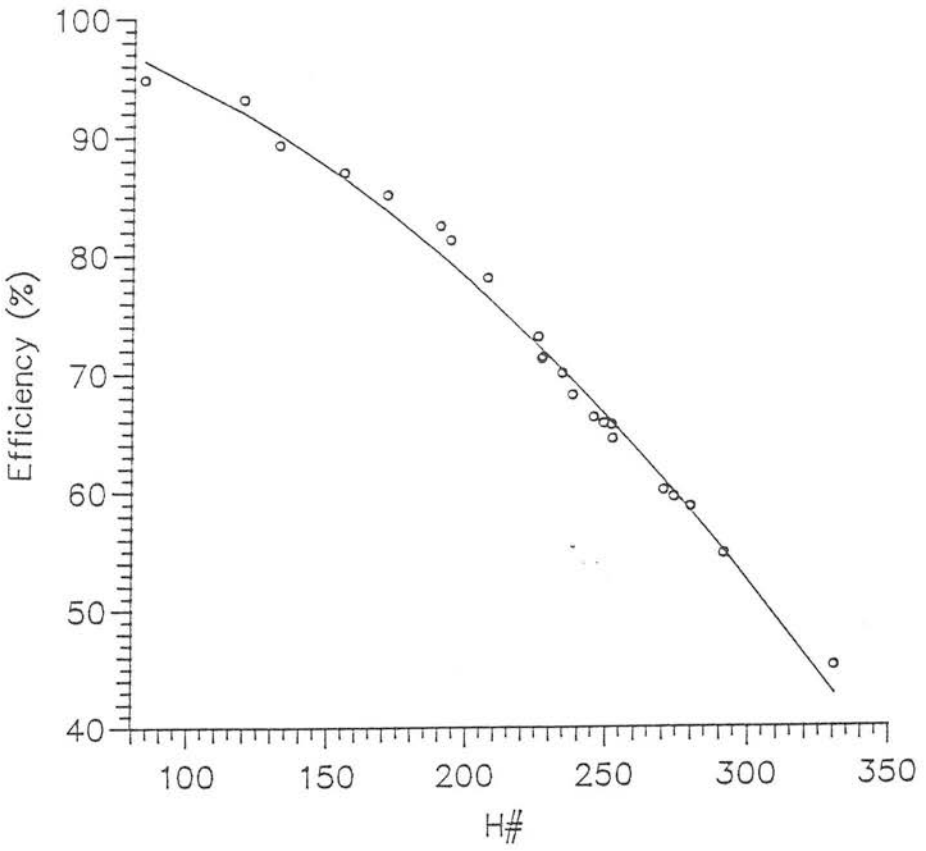
The radioactivity of the samples was measured using a Beckman LS 1701 (Beckman, UK) scintillation counter. Scintillation fluid was prepared by mixing 1l of toluene (BDH), 0.5l triton-100 (BDH) with 6.1g butyl-PBD (2-(4'-tert.-Butylphenyl)-5-(4"-biphenylyl)-1,3,4-oxadiazole) (BDH). The mixture was stirred for 1h in the dark to prepare a uniform solution. The scintillation cocktail was stored at room temperature in the dark until required.

Radioactive spots on TLC were scraped off and added to 5ml of scintillation fluid and counted. Radioactivity in the cell residue was measured by mixing 0.03g dw with 5ml of scintillation cocktail together with fumed silica (BDH), filling half of the volume of the vial, to produce a uniform suspension of finely powdered cell residue.

Radioactivity of the samples was recorded as counts per minute (CPM). The units were converted to disintegrations *per* minute (DPM) using a quench curve obtained

by measuring known activities of [U-^{14}C]toluene (Amersham) quenched by the addition of increasing amounts of acetone (0-500µl) in 5ml of scintillation fluid. Different quench values were obtained and the efficiency was calculated by dividing the counts obtained with the activity. A quench curve was obtained (see Fig. 2.7) with a regression equation of $Y=102-0.0292X-0.000454X^2$. This equation was routinely used for converting CPM to DPM

Figure 2.7 Quench curve obtained as stated in 2.10.3.3. The line plotted is the regression line (see 2.11) with a correlation coefficient of 0.99. $Y=102-0.03X-0.00045X^2$



2.11 Statistical analysis

All experiments, unless stated otherwise, employed at least three replicates. The means and standard errors of the means were calculated to assess the variation within the group of replicates for each treatment, as described by Swinscow (1987).

Linear regression analysis was carried out to calculate regression for establish calibration curves of protein concentration, and the concentration of the standards used for HPLC analysis. Linear regression analysis was also used to determine the relationship between the number of yellow cells and the concentration of [6]-gingerol in sections of *Z.officinale* rhizome and roots.

Student's *t*-tests were also performed to determine whether comparisons of the means were statistically significant. Data expressed as percentage values were transformed by an arcsin transformation to angular values prior to statistical analysis since they do not conform with a normal distribution (Snedecor and Cochran, 1971). Analysis of variance was also performed to compare differences between several treatments.

CHAPTER 3

EXPERIMENTAL RESULTS

In this chapter the experimental results are presented in eight different sections (3.1-3.8). In 3.1 and 3.2 the tissue distribution and cellular localisation of the phenolic pungent principles [PPPs] in *Z.officinale* plants are described. In 3.3 the development of chromatographic procedures for the separation and quantification of the main pungent principle [6]gingerol is set out. Section 3.4 contains results leading to the establishment of a range of cultures and the regeneration of plants. In the following section (3.5) growth and accumulation of the phenolic pungent principles [PPPs] within an established range of cultures are characterised. In 3.6 the fate of [6]gingerol administered to suspension cultures is studied together with an investigation of the possible glycosylation of PPPs in culture. The effects of the addition of sunflower oil to suspension cultures accumulating the pungent principles were also determined. Finally, in sections 3.7 and 3.8 the biosynthetic pathway leading to [6]gingerol was explored using radioactive putative intermediates administered to rhizome pieces and suspension cultures.

3.1 Studies on the distribution of phenolic pungent principles in *Z.officinale*

The aim of this experiment was to study the distribution of the PPPs in different parts of *Z.officinale* plants.

It has been reported (Winton and Winton, 1939; Purseglove *et al.* 1981; Govindarajan, 1982a) that the oleoresin, which contains the pungent principles of the spice, consists mainly of [6]gingerol and a series of homologues, together with a number of terpenoids which make up the aromatic components of the essential oil. These compounds collectively give the flavour and fragrance of ginger and are generally considered to be only present within the rhizome.

In this experiment attempts were made to discover whether the PPPs were also present in other parts of the plant. Mature plants approximately 3-4 months old and

cultivated in the greenhouse (see 2.1) were divided into four parts: shoot axis, leaves, adventitious roots and rhizome, which were then sliced, freeze dried and powdered as described in 2.3.1. The dry powder was then extracted with acetone (see 2.3.1), in order to determine whether the PPPs were present within the different parts. Samples were prepared and analysed by HPLC (see 2.6).

The results presented in Table 3.1.1 show that the highest amount of these PPPs was present within the rhizome; with a much lower amount within the adventitious roots . However, the aerial parts, which comprise the shoot axis and leaves, did not contain any of these compounds.

Table 3.1.1 Amounts of the major pungent principles of *Z.officinale* (gingerol, shogaol) extracted from dried plant material

	[6]gingerol mg g dw^{-1}	[6]shogaol mg g dw^{-1}	[4]shogaol mg g dw^{-1}
Rhizome	7.20	2.05	1.88
Adv. roots	1.94	0.43	0.30
Leaves	nd	nd	nd
Shoot axis	nd	nd	nd

nd=not detected

In the next section attempts were made to establish the cellular localisation of these phenolic compounds in mature rhizomes, immature rhizomes and adventitious roots.

3.2 Studies on the cellular localisation of the phenolic pungent principles of *Z.officinale*

The aim of this set of experiments was to discover the cellular localisation of the PPPs of ginger. Having confirmed that the distribution of the pungent principles is mainly within the rhizome, and discovered that these compounds are also present, but in much lower amounts in the adventitious roots (see 3.1), it was decided to investigate the cellular localisation of these compounds within a range of tissues.

The different plant materials employed for this investigation were: immature rhizomes from micropropagated ginger plants obtained as described in 2.2.3.3, mature ginger rhizomes 3-4 months old cultivated in the greenhouse under the conditions stated in 2. .1, adventitious roots from mature rhizomes; and suspension cultures of ginger (see 2.2.3.4).

Cryo-sections of the various plant materials, obtained as described in 2.7.1, were examined under the light microscope. In all of the plant material examined yellow-pigmented cells (see Fig. 3.2.1) were observed. In order to establish the nature of these yellow-pigmented idioblasts and whether they are likely to be the repositories of the pungent compounds of ginger, various approaches were undertaken. These included: correlation studies between the number of pigmented cells and the amount of [6]gingerol present; several histochemical studies employing three different reagents; a 10% (w/v) solution of sodium carbonate in water, an aqueous mixture of 1% (w/v) ferric chloride and 0.5% (w/v) potassium ferricyanide and a concentrated solution of Nile red (see 2.7.4), in addition to microspectrophotometry and LTSEM (see 2.7.2; 2.7.3).

3.2.1 Correlation studies between the number of yellow pigmented cells and the amount of [6]gingerol

The number of yellow cells in each cryo-section was determined after addition of the sodium carbonate solution, which intensifies the colour and shifts it toward the red thus making counting easier, although the number of coloured cells does not change. The phenolic pungent compounds, including [6]gingerol, were then extracted with 40-45ml acetone (see 2.3.1) from 30 cryo-sections, after observation under the microscope, together with the remaining unsectioned material. The samples were then prepared and analysed using HPLC under the conditions described in 2.6. Results presented in Fig. 3.2.2 show that the number of pigmented cells was highest in tissue from mature ginger rhizomes, lower in micropropagated rhizomes and even lower in cryo-sections taken from adventitious roots. Similar cell types were also observed at very low density in cultured suspended cells although no cell counts were performed using this material. Furthermore, the amounts of [6]gingerol recorded show a similar pattern; the highest amount was recorded from mature rhizomes, with lower amounts in micropropagated rhizomes and in adventitious roots (see Fig. 3.2.2). From this it can be seen (see Fig. 3.2.2) that there is a correlation between the number of yellow cells and the amount of [6]gingerol with a regression coefficient of r=0.782. It was also observed that the intensity of the yellow colour, as well as the size of these cells, varied between cells in the same cryo-section suggesting different stages of cell development. It is probable that a higher regression coefficient would have been expected if the cells had been uniform. The correlation presented here suggests that the site of accumulation of [6]gingerol lies within these pigmented cells.

Figure 3.2.1 Photograph of a cryo-section of tissue from a 3-4 month old ginger rhizome grown under the conditions stated in 2. .1. Observe the presence of a yellow-pigmented cell (idioblast) surrounded by cells with no yellow contents. (od=oil drop, uc=unpigmented cell, yc=yellow cell). Bar=50μm.

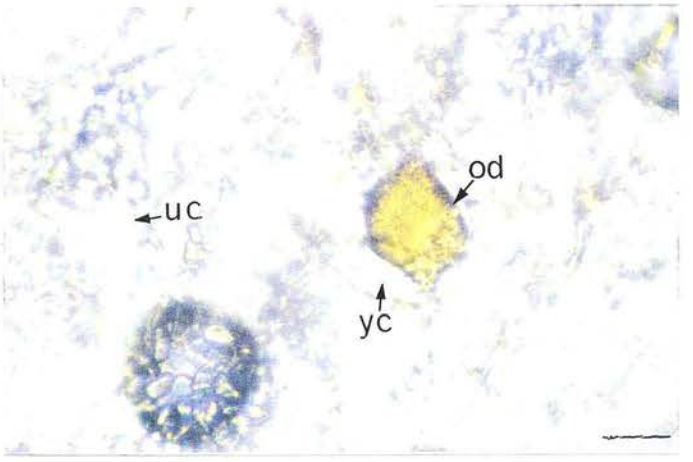
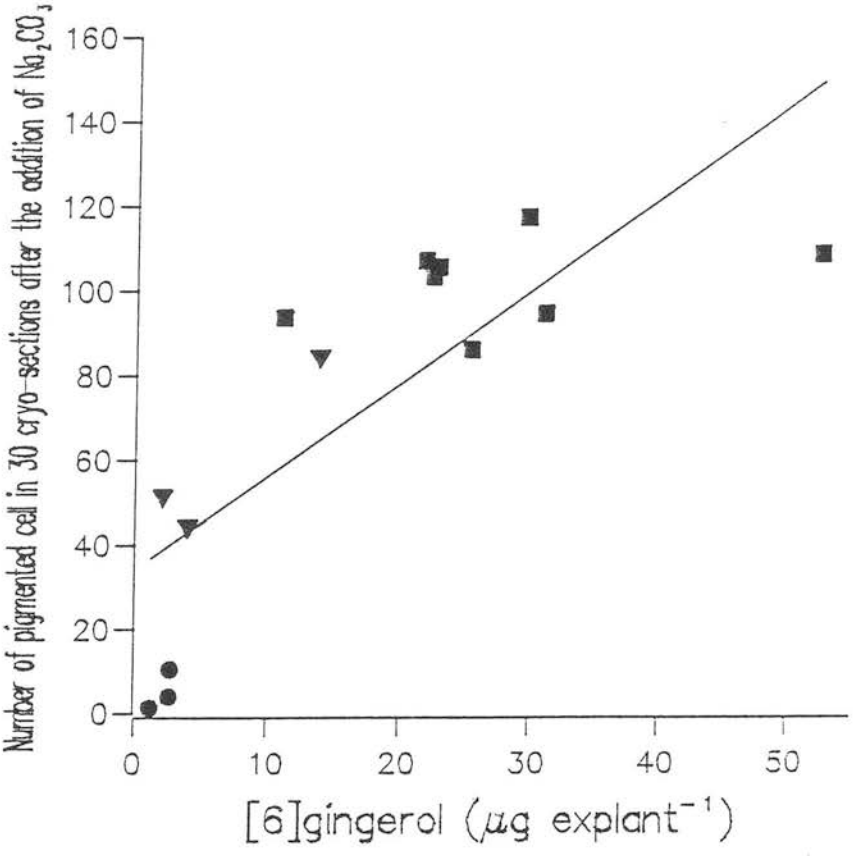


Figure 3.2.2 Correlation between the average number of yellow cells in 30 cryo-sections after the addition of 10% sodium carbonate solution and the amount of [6]gingerol present within the sections and the remaining unsectioned material. Regression equation Y=2.2X+34.3, regression coefficient r=0.782.

(■) cryo-sections from mature rhizome, (●) cryo-sections from adventitious mature roots, (▼) cryo-sections from micropropagated rhizomes



3.2.2 Histochemical studies of cryo-sections of *Z.officinale*

3.2.2.1 Studies employing a solution of sodium carbonate

Having determined the correlation between [6]gingerol content and number of pigmented cells the next stage was to identify the nature of the compounds in these coloured cells using several histochemical approaches. As described previously it was observed that the application of sodium carbonate (see 2.7.4) to the cryo-sections intensified and changed the colour from yellow to orange-red. The results from microspectrophotometric measurements of cryo-sections (see 2.7.3) before and after the application of sodium carbonate (see Fig. 3.2.3a-b) clearly show a shift in absorption from 420nm to 455nm.

In a parallel experiment 3-4 drops of sodium carbonate were applied to 300µl of a concentrated solution of [6]gingerol (193.75µg ml^{-1}), but no shift in absorbance was observed with the maximum remaining at 282nm. Therefore, it can be concluded that the shift in absorbance is due to other secondary compounds present within these cells, which may include flavonoid-like compounds since the addition of sodium carbonate to flavonoids extracted from the flowers of four different species *Rosa canina, Senecio cineraria, S.jacobaea and Verbascum blattaria* (see section 2.4) produced a similar shift in absorbance to that recorded microspectrophotometrically (see Table 3.2.1). It is also probable that curcumin derivatives known to be present in the ginger rhizome produce a similar shift (Harvey, 1981; Tonnensen and Karlsen, 1983).

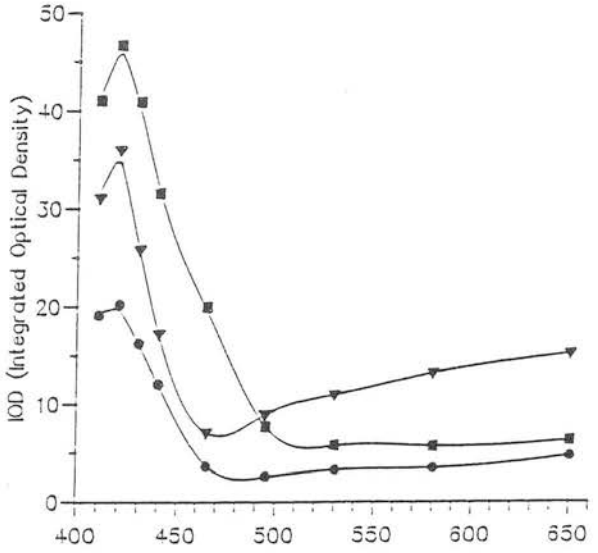
3.2.2.2 Studies employing a solution of ferric chloride-potassium ferricyanide

The application of an aqueous mixture of 1% ferric chloride and 0.5% potassium ferricyanide (see 2.7.4) to the cryo-sections shows that apart from cell walls only cells with yellow contents stained intensely blue (see Figs. 3.2.4a-b). This result demonstrates the presence of compounds of a phenolic nature within the yellow

compartment and bearing in mind the results from the correlation studies, it is suggestive that [6]gingerol is also present, besides flavonoid-like compounds and some curcumin derivatives.

Figure 3.2.3 Microspectrophotometric measurements of three individual yellow-pigmented cells in a cryo-section. (a) before the addition of 10% sodium carbonate solution showing a maximum at 420nm. (b) after the addition of 10% sodium carbonate solution showing a maximum at 455nm.
Each symbol represents a different cell.

(a)



(b)

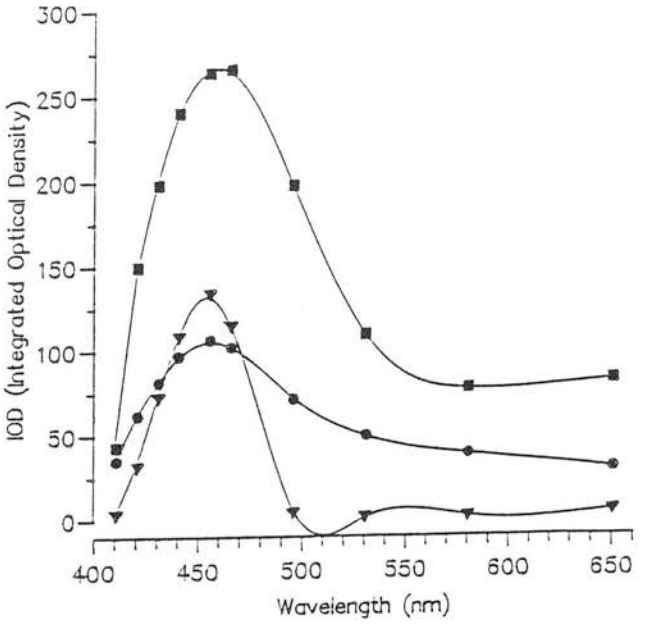
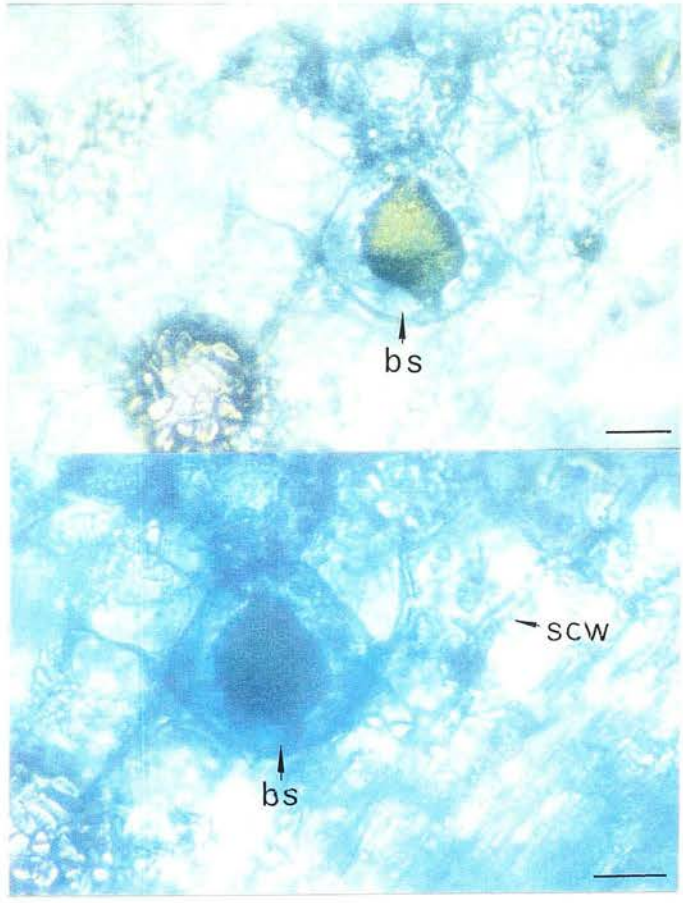


Figure 3.2.4 A cryo-section from a *Z.officinale* rhizome **(a)** observe the same yellow-pigmented cell as in Fig. 3.2.1 photographed 30sec after the addition of a mixture of 1% ferric chloride and 0.5% potassium ferricyanide. A blue colour, indicating the presence of phenolic compounds, begins to develop within the yellow compartment as well as in some of the cell walls, **(b)** same cell photographed after 4min. Observe the blue colour has become very intense within the former yellow cell. (bs=blue staining, scw=stained cell wall). Bar=50μm.



3.2.2.3 Studies employing a solution of Nile red

Considering the toxicity of free phenolic to plant cells, (Brown, 1981) the strategy of accumulation of these phenolics in the idioblasts was investigated. Results from the use of Nile red (see 2.7.4) revealed the lipidic nature of the cell contents which is manifested as red fluorescence (see Figs. 3.2.5a-b). It is also shown in Fig. 3.2.5 that cells with no yellow content did not react with this reagent, thus indicating that a range of secondary compounds are stored within lipids in this type of cell together with components of the essential oil of the spice.

3.2.3 Low temperature scanning electron microscopy of *Z.officinale* rhizome

Further evidence of the presence of lipid material in these specialised oil cells is provided using a freeze etching technique which allows differentiation of organelles and membranes after the removal of the aqueous content (Robards, 1991). LTSEM samples of ginger rhizome were prepared as described in 2.7.2. Results from Figs. 3.2.6a-d show the appearance of the yellow cells with a conspicuous oil drop (see above results with the use of Nile red) lacking a segregated cytosol. Conversely, cells with no oil contents show a clear segregated cytosol into zones due to the presence of water.

All the results presented in this section support the view that the accumulation of gingerol, and possibly other PPPs, together with flavonoid-like compounds and curcumin derivatives lies within these yellow-pigmented cells in the oil. These cells also contain the essential oil of the spice suggesting the presence of only one cell type as a storage compartment for this range of compounds.

In the next section an attempt was made to devise a chromatographic method for the separation of [6]gingerol, the main pungent compound in ginger from the other PPPs.

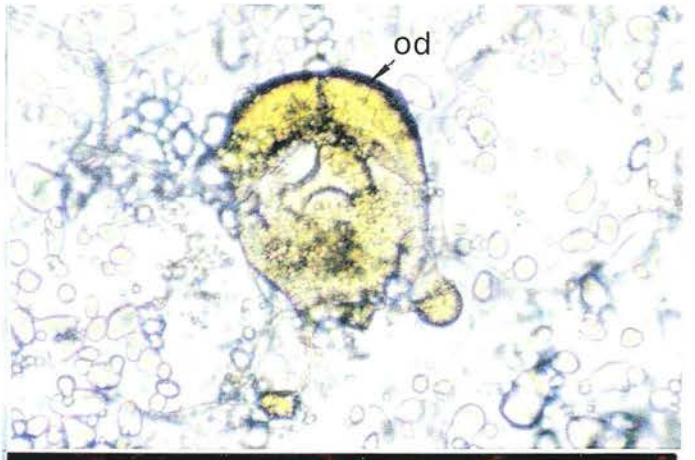
in pooled extracts

Table 3.2.1 Absorbance measurements of flavonoids,) from flowers of four different species *Rosa canina, Senecio cineraria, S.jacobaea and Verbascum blattaria*, before and after the addition of Na_2CO_3.

Maximum OD (nm)	
Before the addition of Na_2CO_3	**After the addition of Na_2CO_3**
370, 382, 390, 410	376, 380, 406, 430, 450

Figure 3.2.5 (a) cryo-section showing an individual yellow cell. Note some of the contents appear to have been released during sectioning, **(b)** same cell stained with Nile red and visualised under green light excitation showing intense red fluorescence within the yellow compartment indicating the presence of lipid material. (od=oil drop, fod=fluorescent oil drop). Bar=50μm.

(a)



(b)

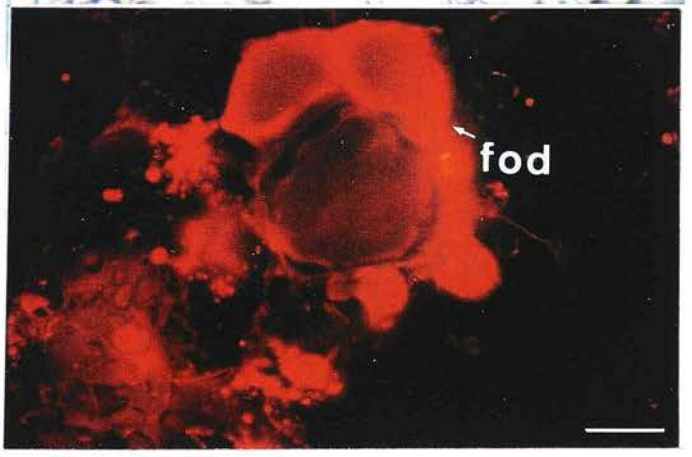
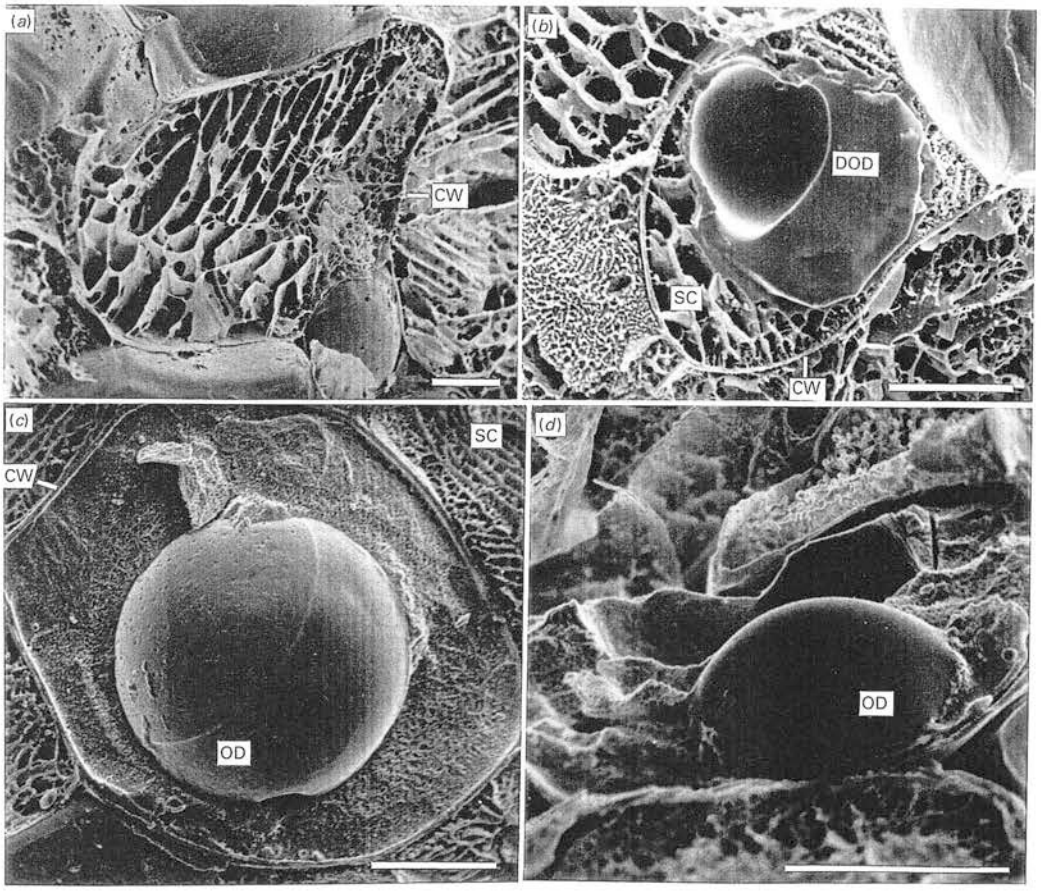


Figure 3.2.6 LTSEM micrographs from a cryo-fractured and etched *Z.officinale* rhizome section **(a)** cell lacking an oil content but revealing an etched cytoplasm segregated into zones, **(b)** cell with a developing oil drop with partially segregated cytosol, **(c-d)** two different cells showing a conspicuous oil drop, note the surrounding cells with no oil content which show a segregated cytosol. (cw=cell wall, dod=developing oil drop, od=oil drop, sc=segregated cytoplasm). Bar=20μm.



3.3 Attempts to isolate the phenolic pungent principles of *Z.officinale* particularly [6]gingerol using two rapid column chromatography techniques

The aim of this set of experiment was to devise a column chromatographic method for the separation of [6]gingerol from a complex mixture of plant compounds.

Twenty g of freeze dried ginger rhizome powder was extracted with acetone as described in 2.3.1. The extract was then evaporated to dryness and re-dissolved in 50ml of 80% (v/v) MeOH-water. This solution was subjected to liquid-liquid extraction (LLE) with hexane 3X (see Fig. 3.3.1) which extracts mainly the waxy materials, volatile oils and some shogaol, this was then analysed on TLC using solvent system I, visualised with Folin-Ciocalteu reagent (see 2.5.1; 2.5.2.2) and then discarded. The remaining MeOH fraction was then partitioned with diethyl ether-water 2:1 (v/v). The aqueous fraction which contains mainly sugars with the PPPs largely present in the ether fraction (see Fig. 3.3.2) was then evaporated to dryness, re-dissolved in 10ml of MeOH and stored as the stock solution.

3.3.1 Flash chromatography

The flash chromatography assembly was as described in 2.9.1.

Two ml of stock solution was taken and evaporated under a stream of nitrogen and then re-dissolved in 2ml of solvent system I (toluene-MeOH, 80:5). The sample was then applied to the column, eluted with solvent system I and 20 fractions of a volume of 10ml collected as stated in 2.9.1. The compounds present in each fraction were analysed by TLC using solvent system I, visualisation of the compounds was achieved after spraying with Folin-Ciocalteu reagent (see 2.5.1; 2.5.2.2).

Although it has been reported that compounds which differ in R_f value by only 0.15 can be satisfactorily separated using flash chromatography, the results presented in Fig. 3.3.3a indicate that there was no separation of the gingerols from the shogaols, the main pungent compounds in ginger, and these compounds appear together in fractions 5 to 7.

Figure 3.3.1 Scheme of liquid-liquid extraction (LLE) of an acetone extract from freeze dried ginger rhizome powder

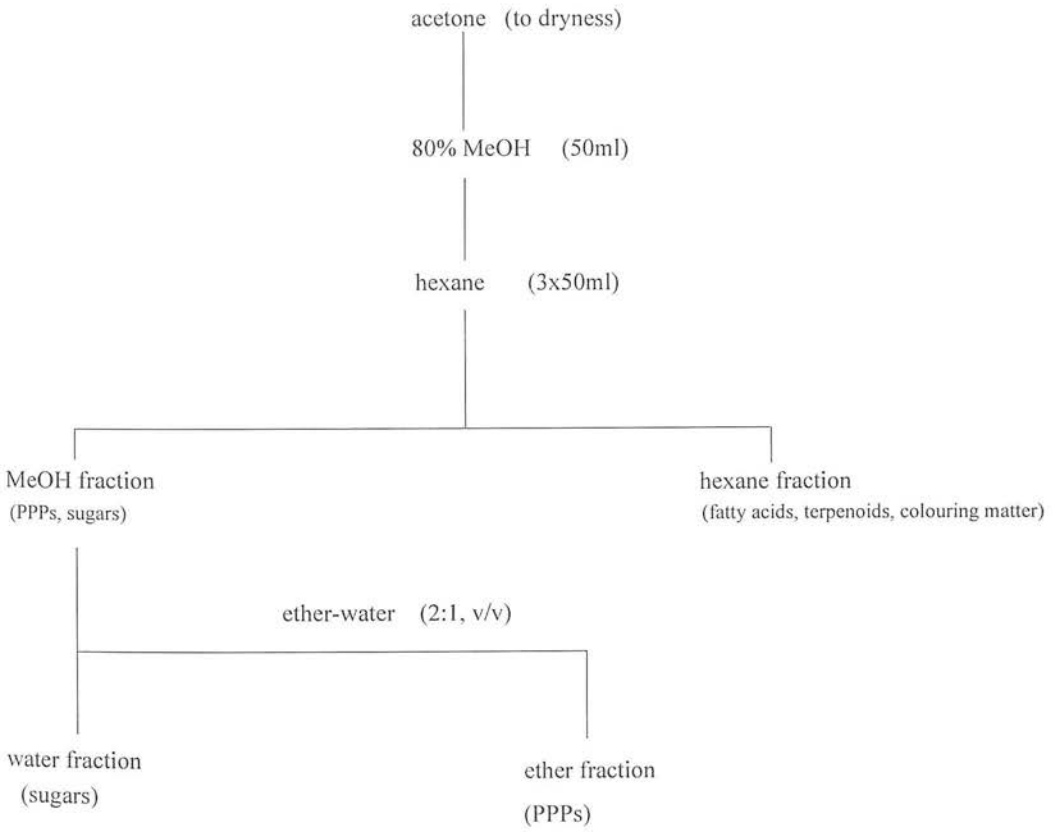
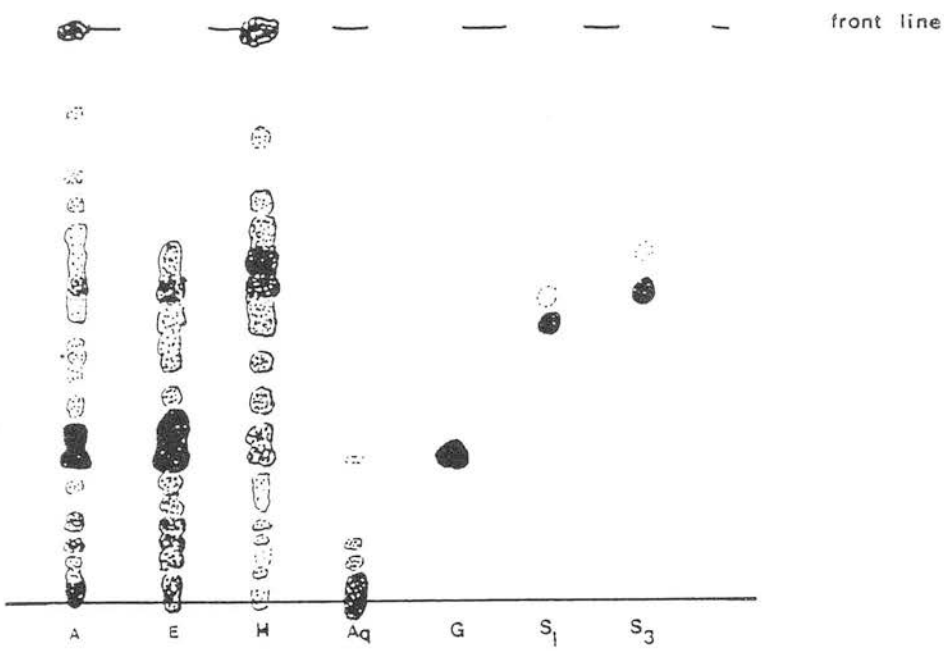


Figure 3.3.2 TLC of the fractions obtained from the LLE. A=total acetone extract, E=ether phase, H=hexane phase, Aq=aqueous phase, G=[6]gingerol, S_1, S_3= shogaol homologues. TLC was developed using solvent system I and visualisation of the compounds was achieved after spraying with Folin-Ciocalteu reagent (see 2.5.1; 2.5.2.2).



Different lengths of the bed column (10-15cm) and different flow-rates (50-100mm per minute) were also tested, as well as the solvent system hexane-diethyl ether (30:70) used in vacuum chromatography, which produced clear separation of the gingerols (see Figs. 2.5 and 3.3.4). However, less satisfactory results were obtained as all the PPPs appear in fractions H_2-H_4 (see Fig. 3.3.3b).

3.3.2 Vacuum chromatography

Vacuum chromatography was performed as stated in 2.9.2. The solvent system employed was initially 25ml hexane followed by 20 aliquots of 25ml of solvent mixtures with increasing polarity comprising hexane-diethyl ether and diethyl ether-MeOH as shown in Fig. 2.5. The volume of the collected fractions was reduced to 10ml under a stream of nitrogen before the samples were loaded onto TLC (see 2.9.2). Using this technique, the main pungent compounds of ginger, the gingerols, are almost completely separated from the shogaols, present in fractions 8 and 9 (see Fig. 3.3.4). It appears that the solvent gradient plays a major part in this chromatographic technique. Moreover, isocratic elution employing solvent system I was also tried; however, a satisfactory separation was not achieved (see Fig. 3.3.3b, fractions T_1-T_5). It seems unlikely that the method of application of the sample to the column has any effect on the efficiency of the separation because the results illustrated in Fig. 3.3.3b (fractions T_1-T_5) show that the separation obtained using an isocratic system was inferior to that with a gradient with the same loading arrangements used for both solvent systems. Therefore, it appears that the solvent gradient generates the satisfactory separation required.

In the previous three sections (3.1-3.3) it has been established the distribution and cellular localisation of the PPPs together with the development of a chromatographic method for the separation of gingerol the main pungent compound in ginger. In the

next section a range of cultures was established to investigate the synthesis and accumulation of the PPPs in ginger.

Figure 3.3.3 (a) TLC of the 10ml fractions collected using flash chromatography performed with solvent system I. **(b)** TLC of the 10ml fractions obtained using flash chromatography with hexane-diethyl ether (30:70) as solvent system, fractions H_2-H_4 and the 10ml fractions collected using vacuum chromatography when an isocratic solvent system toluene-MeOH (80:5) was used, fractions T_1-T_5. Numbers indicate the different fractions collected, E=ether phase, G+S=[6]gingerol and shogaol. Only fractions which seem to contain the compounds under investigation were loaded onto the TLC plate

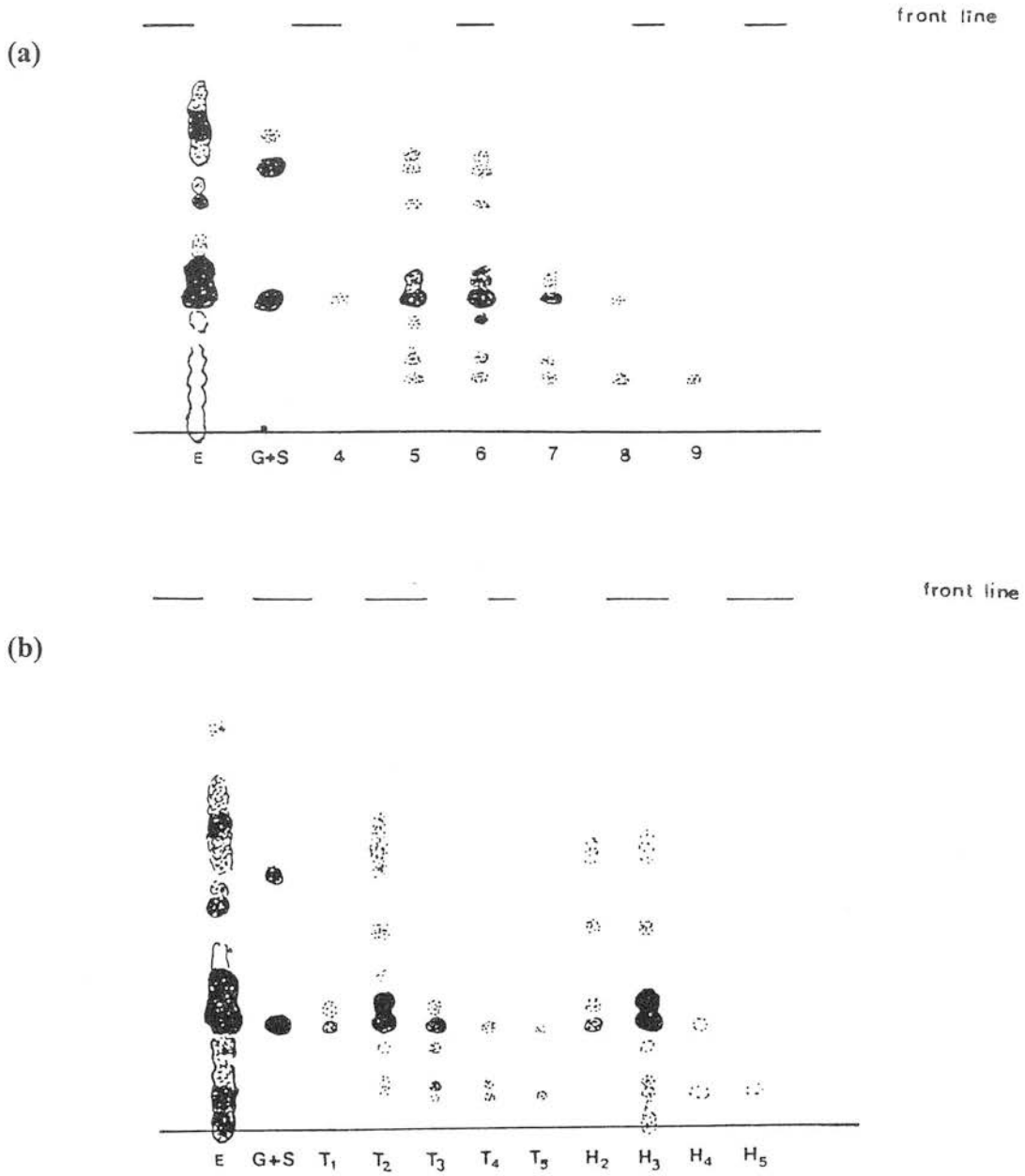
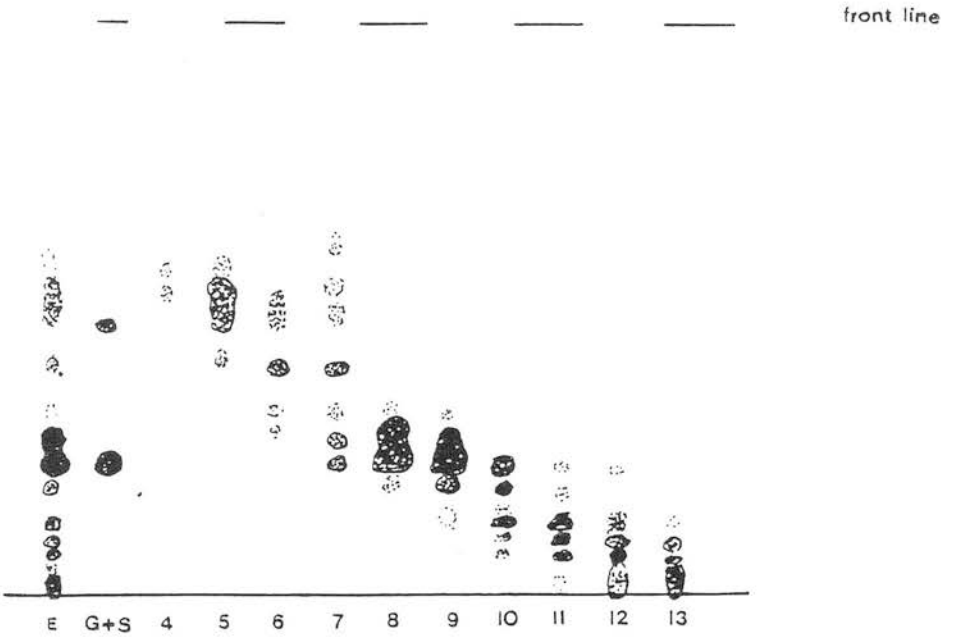


Figure 3.3.4 TLC of the 10ml fractions obtained using vacuum chromatography when a solvent gradient was employed (see Fig. 2.5). Numbers indicate the different fractions collected, E=ether phase, G+S=[6]gingerol and shogaol. Only fractions which seem to contain the compounds under investigation were loaded onto the TLC plate



3.4 Establishment of *Z.officinale* cultures

3.4.1 Initiation and maintenance of callus cultures

The techniques of plant cell and tissue culture have been widely developed and there are many publications on the growth, metabolism and differentiation of tissue cultures of dicotyledonous. However, studies with monocotyledonous plants are less common because of the difficulties encountered in the induction of callus and in maintaining satisfactory growth (Cresswell, 1991). Nevertheless, despite the difficulties some progress has been achieved and various monocotyledonous plants have successfully been cultivated *in vitro eg. Asparagus officinale* (De Vries-Paterson *et al.*, 1992), *Allium sativum* (Malpathak and David, 1986), *Oryza sativa* (Cao *et al.*, 1992), *Zea mays* (Green and Rhodes, 1982), and *Zingiber officinale* (Ilahi and Jabeen, 1987; Bhagyalakshmi *et al.*, 1988; Babu *et al.* 1992; Kackar *et al.*, 1993).

Generally, a high level of an auxin-type PGR combined with a low level of cytokinin, is necessary for the successful culture of monocotyledons together with an adequate basal nutrient medium (Schenk and Hildebrandt, 1972). *In vitro* cultures of ginger have been chiefly directed towards the development of micropropagation methods (Hosoki and Sagawa, 1977; Ilahi and Jabeen, 1987 and 1990; Noguchi and Yamakawa, 1988) to provide large numbers of parasite free plantlets from a single explant *eg.* the elimination of nematodes from the ginger rhizome as reported by De Lange *et al.* (1987) and to achieve crop improvement via *in vitro* culture (Malamug *et al.*, 1991; Babu *et al.* 1992)

In the previous sections (3.1, 3.2) it was shown that the major localisation of the pungent phenolics in ginger lies in the rhizome of the plant, with a much lower level in the adventitious roots, and none in the aerial parts of the plant. In order to investigate the production of the pungent principles of ginger in tissue culture, it was required to initiate and establish callus cultures from growing ginger rhizomes. The aim of this experiment was to induce callus cultures of ginger.

At first callus was initiated from small, regular explants (Caplin, 1963), taken from the cortex and medulla of surface sterilised ginger rhizomes (2.2.2.2).

Twenty five cylindrical inocula, obtained as described in 2.2.3.1, were placed individually on the surface of 10-12ml of several solidified culture media (see Table 3.4.1 and 3.4.2) contained in sterile Petri dishes 5cm in diameter. The dishes were sealed with Parafilm and then placed in an environmentally controlled culture suite (see 2.2.3.1). The cultures were monitored at regular intervals over a period of 60d for the appearance of callus and incidence of contamination .

Table 3.4.1 Composition of the culture media employed for the initiation of *Z. officinale* cultures. SH basal salts medium (Schenk and Hildebrandt, 1972).

SHA	SHB	SHC	SHD	SHE
3% sucrose	3% sucrose	3% sucrose	3% sucrose	3% sucrose
0.5mgl^{-1}2,4-D	1mgl^{-1}2,4-D	2mgl^{-1}2,4-D	3mgl^{-1}2,4-D	5mgl^{-1}2,4-D
0.1mgl^{-1}Kin	0.1mgl^{-1}Kin	0.1mgl^{-1}Kin	0.1mgl^{-1}Kin	0.1mgl^{-1}Kin
1mgl^{-1}CPA	1mgl^{-1}CPA	1mgl^{-1}CPA	1mgl^{-1}CPA	1mgl^{-1}CPA
pH 5.8	pH 5.8	pH 5.8	pH 5.8	pH 5.8
0.8% agar	0.8% agar	0.8% agar	0.8% agar	0.8% agar

Table 3.4.2 Composition of the culture media employed for the initiation of *Z.officinale* cultures. MS basal salts medium (Murashige and Skoog, 1962).

1/2MSA	1/2MSB	1/2MSC
3% sucrose	3% sucrose	3% sucrose
0.5mgl^{-1} 2,4-D	1mgl^{-1} 2,4-D	2mgl^{-1} 2,4-D
0.5mgl^{-1}BAP	0.5mgl^{-1}BAP	0.5mgl^{-1}BAP
pH 5.8	pH 5.8	pH 5.8
0.8% agar	0.8% agar	0.8% agar

A high percentage of contamination (30-40%) was observed during the culture period. Similar high levels of contamination up to 50% have been reported earlier by Hosoki and Sagawa (1977). Under the culture condition reported here, explants became whitish in colour and swelled during the culture period without the appearance of callus. It would appear, considering the number of different media tested, that the source of plant material used was not appropriate for the initiation of callus and that the high level of contamination observed may have been due to an endogenous contaminant within the plant tissue which remained after surface sterilisation.

In the next experiment, a different source of plant material was employed. A number of authors have reported the use of emerging axillary buds from ginger rhizomes for micropropagation of the plant (Bhagyalakshmi *et al.*, 1988; Noguchi and Yamakawa, 1988; and Malamug *et al.*, 1992;) The plant material used in this study was removed from 10-15d emerging axillary buds from ginger rhizomes grown under the conditions stated in 2.1. Axillary buds were excised from the rhizome and then surface sterilised (see 2.2.2.2). The buds were then cut transversely into slices 3mm thick and explants taken from these slices with a cylindrical cork borer 4mm in diameter. Twenty five cylindrical explants were placed individually on the surface of 10-12ml of several solidified culture media (see Table 3.4.1 and 3.4.2) contained in sterile Petri dishes 5cm in diameter which were sealed with Parafilm and then placed in an environmentally controlled culture suite (see 2.2.3.1). The cultures were monitored throughout a period of 60d for the appearance of callus and incidence of contamination. Under these culture conditions, the percentage of contamination was lower than in the previous experiment, when explants taken from the cortex and medulla of ginger rhizomes were used. The highest percentage of contamination was 40% in culture medium SHE with an average of 20% for cultures with SH as basal salts (see Table 3.4.3). Using this source of plant material, callus induction was evident in some of the inocula after two to three weeks. However, variation in the

response was observed with the media tested. A lower incidence of callus induction was recorded in inocula cultured on nutrient media (SH). Nevertheless, inocula cultured on SHB medium showed a relatively high induction rate (see Table 3.4.3) which was not significantly different than that obtained with 1/2MSA and 1/2MSB media at P=0.05 although there was difference at P=0.01. However, callus formed on this medium (SHB) was hard. When these hard pieces of callus were sub-divided with a scalpel and subcultured onto fresh medium they became friable after several passages. On the other hand, inocula cultured on growth media with MS basal salts gave the highest rate of callus induction of the eight media tested (see Table 3.4.3). Inocula on media 1/2MSA and 1/2MSB showed higher callus induction than those on medium 1/2MSC. The callus produced on these media was slightly more friable than that produced on SH media. This callus was cut into small pieces and sub-cultured every 5-6 weeks. After several subcultures the growth of callus on 1/2MSC medium slowed down and the mass of callus became brown and appeared to be necrotic, suggesting perhaps a toxic effect of the 2.4-D concentration used. It was also observed, as for calli growing on media with SH basal salts, that callus became more friable after several passages onto fresh medium. Subsequently sub-culture was made onto fresh 1/2MSB and SHB media as these appeared to be more favourable for sustained growth.

Table 3.4.3 Response of the explants to callus induction after 60d,

25 inocula per treatment

Medium	Contamination (%)	Callus induction (%)	Appearance of callus
SHA	20	10	h with hls
SHB	12	27.27	h
SHC	12	18.18	h with hls
SHD	16	14.28	h
SHE	40	5	h but mf
1/2MSA	12	31.81	h
1/2MSB	8	47.82	h
1/2MSC	16	19.04	h but mf with hls

callus induction (%)=No. inocula showing callus/No. clean inocula x 100

contamination (%)=No. contaminated inocula/total inocula x 100

h= hard hls= hair like structures mf= more friable

3.4.2 Induction of plant regeneration

After the establishment of callus cultures (see 3.4.1), the induction of plant regeneration from cylindrical inocula was undertaken to facilitate studies on the changes in PPPs during induced organogenesis.

It has been reported that various combinations of the PGRs cytokinin and auxin promote either shoot or root initiation. A high auxin-cytokinin ratio promotes root

formation. Conversely, a low auxin-cytokinin ratio induces shoot formation (Warren, 1991).

Two different culture media were tested for the regeneration of *Z.officinale* plants (see Table 3.4.4).

Table 3.4.4 Composition of two culture media employed for the regeneration of *Z.officinale* plants

MS 5/1 culture medium	**MS 3/0.5 culture medium**
M+S	M+S
3% sucrose	3% sucrose
5 mgl^{-1} BAP	3 mgl^{-1} BAP
1 mgl^{-1} NAA	0.5 mgl^{-1} NAA
0.8% agar	0.8% agar
pH 5.8	pH 5.8

Seventy five surface sterilised cylindrical inocula, obtained from 10-15d old emerging axillary buds as described in 2.2.3.1, were inoculated individually onto 10-12ml of each solidified culture media contained in Petri dishes 5cm in diameter, which were then double sealed with Parafilm and placed in an environmentally controlled culture suite (see 2.2.3.1).

The results displayed in Table 3.4.5 show the extent of contamination and induction of organogenesis in explants cultured on two media after 40d. It can be seen that the contamination rate was very similar for both treatments. Response recorded as formation of roots was higher on MS 5/1 medium although there was no statistical difference (at P=0.05) (see Table 3.4.5). The induction of shoots was similar on the two media; however, plantlet formation was statistically higher on MS 3/0.5 (at

P=0.05). Moreover, callus formation was more evident on MS 5/1 medium although there was no statistical difference at either P=0.05 or P=0.1 (see Table 3.4.5), although at this later probability level the results just missed the difference. Therefore, it would appear that under these culture conditions MS 3/0.5 was more effective than MS 5/1 showing a higher plantlet formation (see Table 3.4.5 and Fig. 3.4.1a-b).

Table 3.4.5 Effect of culture medium on the induction of morphogenetic structures and plantlets from explants of *Z.officinale* after 40d

	Medium	
	MS 5/1 culture medium	**MS 3/0.5 culture medium**
Contamination	28.0% **a**	24.0% **a**
Root induction	26.6% **a**	13.3% **a**
Shoot induction	26.6% **a**	26.6% **a**
Plantlet induction	6.60% **a**	33.3% **b**
Callus formation	40.0% **a**	26.6% **a**

Values within a row not followed by the same letter differ significantly (P=0.05) by X^2-test analysis (df 1)

Figure 3.4.1a-b Appearance of explants after 40d culture **(a)** on MS 5/1 medium and **(b)** on MS 3/0.5 medium. It can be seen that inocula cultured on medium MS 3/0.5 developed shoots and roots whilst inocula on medium MS 5/1 developed roots and callus

(a)

(b)

3.4.3 Initiation and maintenance of *Z.officinale* suspension cultures

In previous experiments (see 3.4.1) callus cultures were established; and it was shown that those media based on MS appeared to be more effective for the initiation and growth of callus than SH medium. The following experiments were now conducted to establish suspension cultures.

Suspension cultures were initiated using two different liquid media, 1/2MSB and SHB. These media had been employed initially for the initiation of callus cultures (see Tables 3.4.1; 3.4.2) but with added agar. Initially these calli were hard but gradually, after several passages onto fresh medium, became more friable.

Due to the small amount of friable callus available, small Erlenmeyer flasks of 125ml capacity containing 20ml of sterilised liquid medium were employed. Approximately 0.25g of the most friable callus was transferred aseptically into the same liquid medium (1/2MSB, SHB) contained in 125ml Erlenmeyer flasks, which were then sealed with a double layer of aluminium foil and placed on an orbital shaker in an environmentally controlled culture room at 25±2°C, in a continuous light (see 2.2.3.4).

Because of the small amount of friable callus available the inoculation of large numbers of flasks was not possible, this prevented detailed growth analysis at this stage and therefore only visual observations were made.

Under these culture conditions it was observed that after a period of 25d, there was no apparent increase in biomass with either of the media. This could be due to the small amount of initial inoculum (*ca.* 0.25g) which reduced cell-cell contact and slowed down cell division and growth (Street, 1977). Subsequently, a larger amount of friable callus (*ca.* 0.5g) was used to initiate another set of cultures. This friable mass of callus was taken from two solidified cultures of 1/2MSB and SHB and placed aseptically into two different liquid media, 1/2MSB and SHB, in 125ml Erlenmeyer flasks and incubated on a shaker as described in 2.2.3.4.

After 25d in culture an increase in cell biomass was observed in 1/2MSB and some green differentiated structures were also apparent. Some growth was also recorded in SHB medium and a few green differentiated structures were also observed as in 1/2MSB.The cultures were then left to grow and to build up sufficient cell biomass to enable a larger number of flasks to be set up so that culture growth could be measured as PCV (see 2.2.4.3).

The next experiment was performed to study the growth of cells in two liquid culture media 1/2MSB and SHB. Growth was determined as the increase in PCV (see 2.2.4.3); *ca.* 0.5g wet weight of cells were inoculated into 20ml of 1/2MSB and SHB liquid media in 125ml Erlenmeyer flasks. Determination of PCV was performed on three replicates of each culture taken every 6d for 30d, this was to determine the best growth medium.

The results presented in Table 3.4.6 show that growth was better in 1/2MSB with a 2.27 fold increase in PCV over 30d. A lower increase of 1.29 fold was recorded for SHB medium over 30d.

Table 3.4.6 Changes in the PCV of freshly initiated suspension cultures of *Z.officinale* in two media. Each value is the mean of three replicates. Data were transformed to Arcsin angular values for statistical comparison (see numbers in brackets)

Medium	PCV(%)						Increase
	0d	6d	12d	18d	24d	30d	
1/2MSB	4.3 (11.97±0.08)	4.8 (12.7±0.16)	6.2 (14.4±0.5)	7.3 (15.65±0.67)	9.5 (17.95±0.28)	9.8 (18.24±0.34)	2.27 fold a
SHB	4.4 (12.11±0.16)	4.5 (12.3±0.36)	5.0 (12.88±0.76)	4.8 (12.65±0.15)	5.5 (13.13±0.24)	5.7 (13.77±0.79)	1.29 fold b

a, b significantly different at d 18, 24 and 30 (at P=0.05)

As medium 1/2MSB gave a significantly better result than SHB after 18d in culture (at P=0.05) (see Table 3.4.6), it was decided to use this medium in future experiments with suspension cultures (see 2.2.3.4).

Having established a suitable medium for maintaining suspension cultures it was now necessary to study the kinetics of growth.

3.4.3.1 Investigation of the growth of *Z.officinale* in suspension culture

It has now been shown that suspension cultures (3.4.3) grew better on 1/2MSB than on SHB. Subsequently suspended cells were maintained as cultures in 40ml of liquid medium contained in 250ml Erlenmeyer flasks. In this experiment several growth parameters including PCV, fw, dw and pH were determined during a culture cycle. The effect of a higher inoculum size as well as the concentration of 2,4-D in the medium was also studied.

Cultures were initiated by inoculating 0.5g wet weight of filtered suspended cells (3.4.3) into 40ml of 1/2MSB liquid medium. The increase in cell biomass was assessed by recording PCV, fw and dw (see 2.2.4.3; 2.2.4.1 and 2.2.4.2) every 5d

over a period of 70d. The pH of the medium was also determined at these intervals using a pH meter. The results are presented in Figs. 3.4.2a-d. It can be seen that there was no significant change in PCV during the first 5d (see Fig. 3.4.2a). This was then followed by a steady increase reaching a PCV 4.4x the initial value after 20-25d. PCV then remained relatively constant until d 50 (stationary phase) and then decreased probably due to the depletion of nutrients in the medium. The changes in fw presented in Fig. 3.4.2b also show a similar pattern with an initial lag phase lasting for 5d followed by a steady increase up to d 30-35 giving an overall increase of 5.25 fold. After 35d the fw tended to decrease until the end of the experiment. The overall increase in fw over the culture cycle was 5.25 fold. However, the maximum fw produced was only *ca.* 3.7g indicating very slow growth with a low production of cell biomass. Fig. 3.4.3c presents changes in the dw of cultures which show a similar pattern to those described in Figs 3.4.2a and 3.4.2b. with a 4.4 fold increase throughout the culture cycle.

The pH of the cultures was also monitored and these values are presented in Fig. 3.4.2d, from which it can be seen that a variation in pH took place during culture. After autoclaving the pH fell from 5.8 to *ca.* 5.2 and a further drop to 4.4 occurred after inoculation. The pH remained at this level until day 30 when a sharp rise was recorded reaching a value of 5.8, which coincided with the onset of the stationary phase. Even higher increases were recorded in excess of 7.0 when growth had stopped and cells began to die. Variation in pH is probably caused by changes in the ratio of nitrate-ammonia in the nutrient medium, as has been reported with carrot suspension cultures by Veliky and Rose (1973). This is consequent on the depletion of nutrients and the liberation of compounds into the nutrient medium.

Figure 3.4.2a Changes in the PCV of suspended cells cultured in 1/2MSB medium. (*) Each value is the mean of three replicates, (o) each value is the mean of three replicates ± se after Arcsin transformation

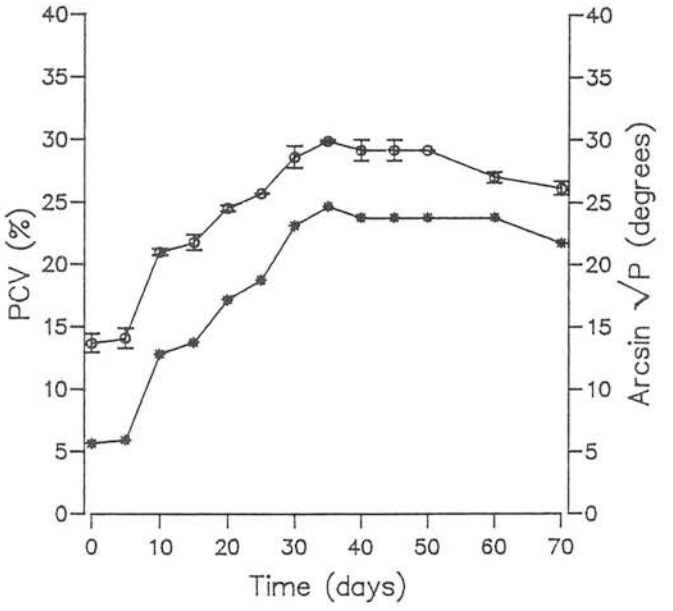
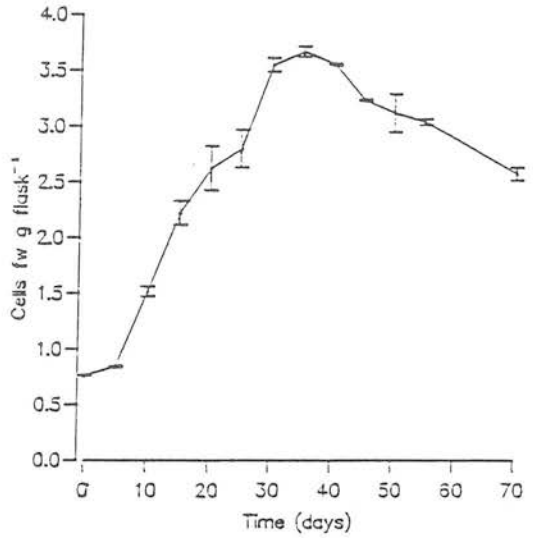
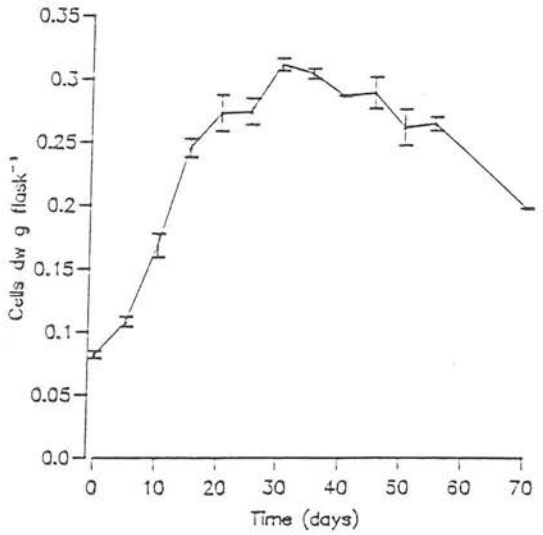


Figure 3.4.2b-d Changes in **(b)** fresh weight (fw), **(c)** dry weight (dw) and **(d)** pH of suspended cells cultured in 1/2MSB. Each value is the mean of three replicates ± se

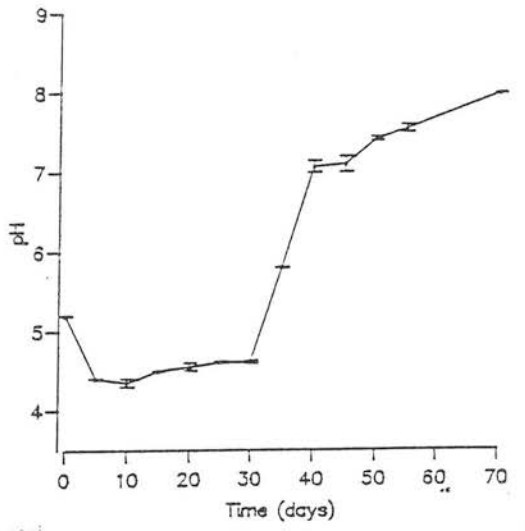
(b)



(c)



(d)



In the next two experiments the effects upon growth of a higher inoculum size and of a lower concentration of 2,4-D are studied.

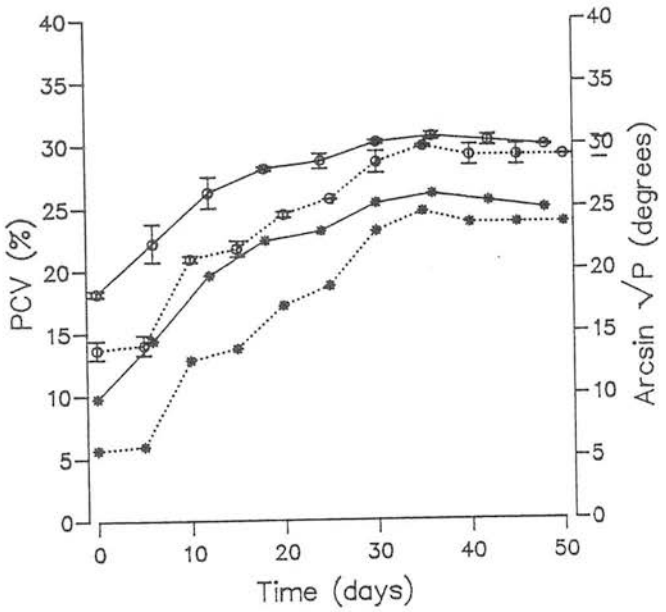
It was established in 3.4.3 and 3.4.3.1 that an inoculum size of 0.25g appeared to be inadequate to sustain growth but 0.5g was satisfactory in 1/2MSB. Thus, in order to study the effect of a higher inoculum size upon growth a culture was set up inoculating 1g wet weight of cells. The PCV was recorded every 5d over a 50-55d culture period (2.2.4.3).

Results presented in Fig. 3.4.3a show the absence of a lag phase and PCV of cultures increased immediately after inoculation exhibiting a 2.85 fold rise at d 20. Cultures then showed a further increase of 1.1 fold from d 20 to 40 after which PCV remained fairly constant. These results show that the higher inoculum size of 1g does not produce a significantly greater biomass (see Appendix) than 0.5g which was the inoculum size chosen for subsequent experiments.

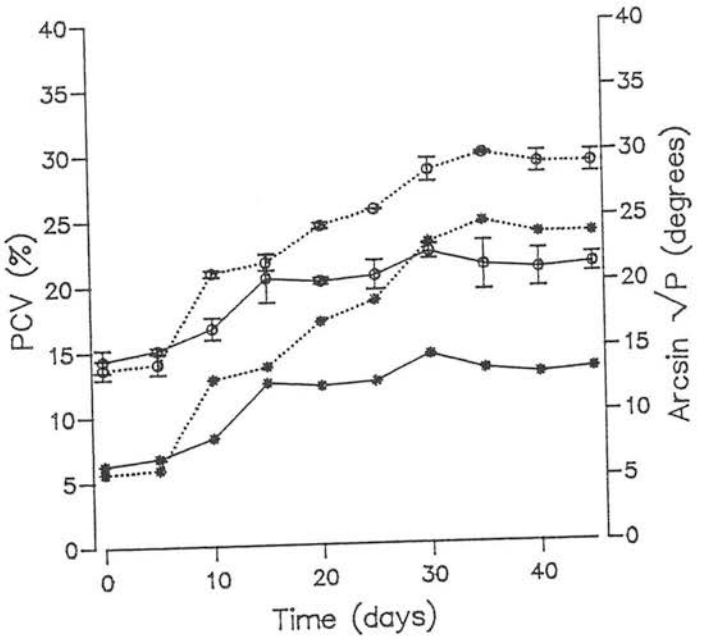
A second set of cultures was prepared using inocula of 0.5g wet weight cells to study the effects of a lower amount of 2,4-D upon growth. 1/2MSA liquid medium (see Table 3.4.2) supplemented with $0.5mgl^{-1}$ 2,4-D was employed. PCV was recorded every 5d throughout the culture cycle to determined the increase in biomass (2.2.4.3), three replicates were used. Fig. 3.4.3b shows the changes in PCV of 1/2MSA cultures. A smooth rise was observed from d 0 to d 10 with a 1.3 fold increase. From d 10 to 15 PCV increased 1.5 fold reaching a high point at d 30. This was followed by a decrease of 1.5 fold when growth ceased. Statistical analysis showed a significant difference between the PCV values of 1/2MSB and 1/2MSA cultures (see Appendix). Thus, it would appear that this culture medium with a lower amount of 2,4-D is less favourable than the initially tested 1/2MSB. Therefore 1/2MSB medium was used as standard liquid medium for further studies.

Figure 3.4.3a-b Changes in **(a)** PCV of suspended cells cultured in 1/2MSB medium with an initial inoculum of 1g (continuous line) and control (1/2MSB with an inoculum of 0.5g) (dotted line), **(b)** PCV of suspended cells cultured in 1/2MSA medium supplemented with a lower amount of 2,4-D ($0.5mgl^{-1}$) with an inoculum of 0.5g (continuous line) and control (1/2MSB with an inoculum of 0.5g) (dotted line). (*) Each value is the mean of three replicates, (○) each value is the mean of three replicates ± se after Arcin transformation

(a)



(b)



In the next section the growth of cultures and accumulation of PPPs in a number of culture systems is presented. Studies were also conducted on the effects of several liquid media on the growth and accumulation of PPPs. Attempts were also made to control the pH.

3.5 Characterisation of growth and accumulation of the phenolic pungent principles of *Z.officinale* in culture

In this section the characterisation of growth and accumulation of PPPs in a range of established cultures is presented.

3.5.1 Characterisation of growth and accumulation of the phenolic pungent principles in callus cultures of *Z.officinale*

Callus cultures of ginger were established on two different solid media 1/2MSB and SHB (see 3.4.1). It was also shown that explants cultured on 1/2MSB produced callus significantly earlier than those on SHB at P=0.01 (no significant difference at P=0.05). In this experiment these media were used to study growth of the cultures by measurements of fresh weight and protein content and the pattern of accumulation of PPPs using HPLC. The proportion of yellow cells, which have been reported to be the repositories of a series of compounds including [6]gingerol, the main pungent principle of ginger, (see 3.2) was also studied over a culture period of 60 d.

Surface sterilised emerging axillary buds from a ginger rhizome (10-15d after planting) were cut transversely into slices of *ca.* 3mm in thickness and 2-3 cylinders removed from each slice with a cylindrical cork borer 4mm in diameter (see 2.2.3.1). Seventy cylindrical inocula were placed individually on the surface of 10-12ml of 1/2MSB or SHB solid media contained in 5cm diameter Petri dishes which were double sealed with Parafilm and placed under standard culture conditions (2.2.3.1).

Three replicates were taken every 10d over a culture cycle of 60d. Determination of fw was performed to assess growth. A rectangular piece of tissue of 2.5x2.0mm and *ca.* 0.02g fw was taken from the middle part of the explants, frozen and the entire tissue sectioned at a thickness of 25-30μm using a cryostat . The number of pigmented cells in each section was determined using a light microscope after the addition of a 10% (w/v) solution of sodium carbonate (see 2.7.1 and 2.7.4). The replicate sections were then put together with the remainder of each explant and the PPPs extracted with acetone (2.3.2.1). Quantitative analysis of the tissue extract was performed by HPLC under the conditions stated in section 2.6. After acetone extraction, the residual dry material was subjected to protein extraction with 0.1N NaOH and the dissolved protein determined (2.2.4.5).

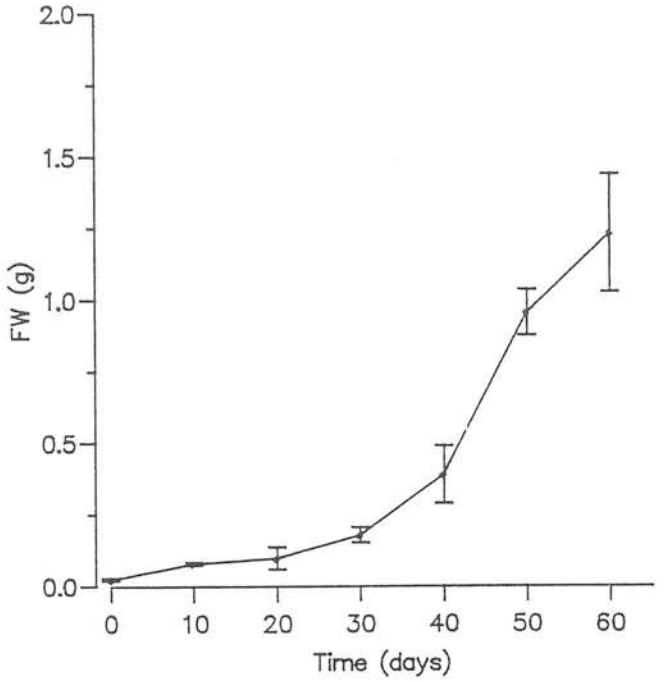
Figs. 3.5.1a-b show the changes in fw of explants cultured on 1/2MSB and SHB media. It can be seen that an increase of 7.2 fold, for explants on both media, occurred from d 0 to d 30. After this initial increase there was a further rise in fw from d 30 to 60 of 6.9 fold on 1/2MSB and 9.5 fold on SHB, but this difference was not significant (at P=0.05) (see Appendix). Thus, it would appear that both culture media support similar patterns of growth. Similar patterns in protein content of both cultures are shown in Figs. 3.5.2a-b which parallel the changes in fw for both cultures (see Figs. 3.5.1a-b). It was observed that cultures did not show a clear stationary phase within the 60d culture period.

The results presented in Figs. 3.5.3a-b show a maximum amount of [6]gingerol and [6]shogaol per g fw at the onset of culture. This was then followed by a gradual decrease throughout the culture period reaching a low value at d 50-60 which coincides with the highest fw and protein content. Thus, it can be observed that there is an inverse relationship between fw, and protein content and the amount of PPPs present. Conversely the results illustrated in Figs. 3.5.3c-d show the changes in the amount of PPPs per explant. It can be seen from Fig. 3.5.3c that the amount of [6]shogaol remained fairly constant throughout the experimental period; however, the

amount of [6]gingerol after a small initial decrease (d0 to d10) showed a clear increase until d 30 (2.25 fold) which preceded a sharp drop on d 40 followed by a steep increase at d 50 reaching a maximum value with an overall increase of 3.13 fold. At d 60 the amount had fallen sharply to reach a value close to that recorded at d 10. It would appear that a variable pattern occurs, perhaps indicating variation within the replicates. The results presented in Fig. 3.5.3d show the changes in the amount per explant of [6]gingerol and [6]shogaol in explants cultures on SHB. As with the results obtained on 1/2MSB medium the amount of [6]shogaol remained almost constant throughout the entire culture period, apart from a small increase at d 60. On the other hand the amounts of [6]gingerol tended to decrease from the onset of culture until d 20, then rose slightly (1.1 fold) by d 30-40. At d 50 there was a drop in the amount of [6]gingerol which was preceded by an overall increase in 1.61 fold at d 60. The amounts of PPPs present in the solidified medium could not be determined due to the great difficulty in the extraction thus, it would appear possible that some PPPs may have been released into the medium. Therefore, it would appear that a lower increase in the amount of [6]gingerol occurred in this medium suggesting that the composition of the basal medium (SH) together with the supplemented PGRs did not favour the accumulation of [6]gingerol.

Figure 3.5.1a-b Changes in the fw of explants cultured on **(a)** 1/MSB medium and **(b)** SHB medium. Each value is the mean of three replicates ± se

(a)



(b)

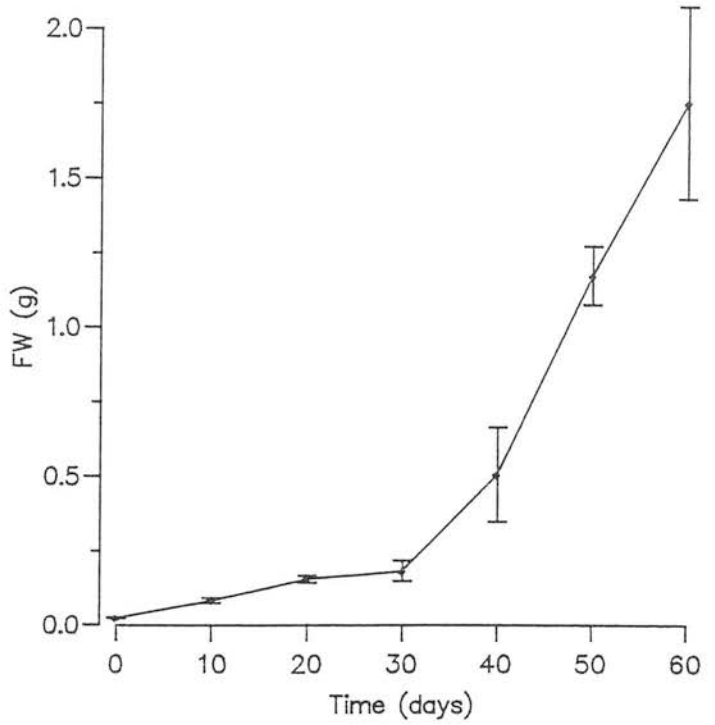
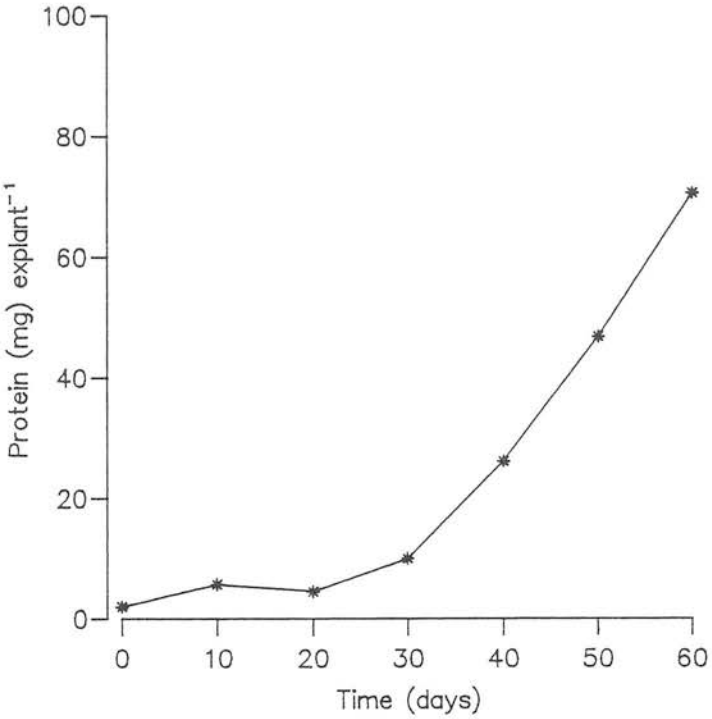


Figure 3.5.2a-b Changes in the protein content of explants cultured on (a) 1/2MSB medium and (b) SHB medium. Each value is the mean of three replicates

(a)



(b)

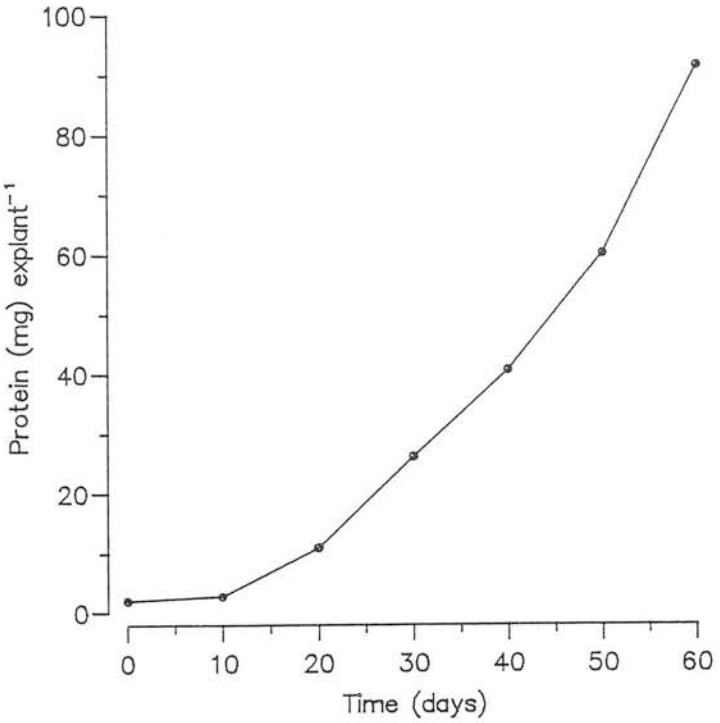
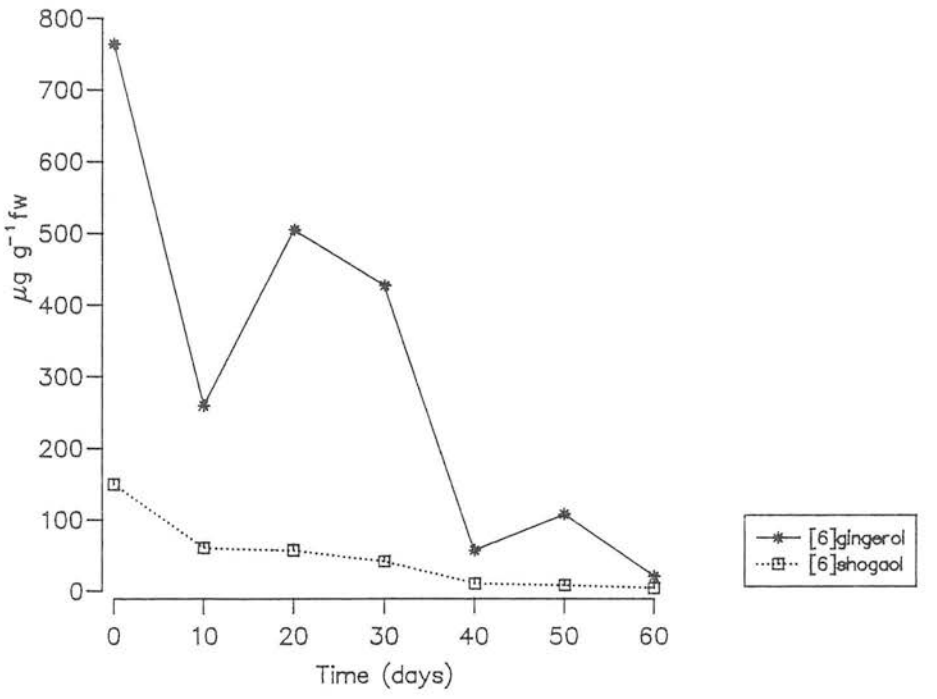


Figure 3.5.3a-b Amount (per g fw) of [6]gingerol and [6]shogaol in explants cultured on **(a)** 1/2MSB medium and **(b)** SHB medium. Each value is the mean of three replicates

(a)



(b)

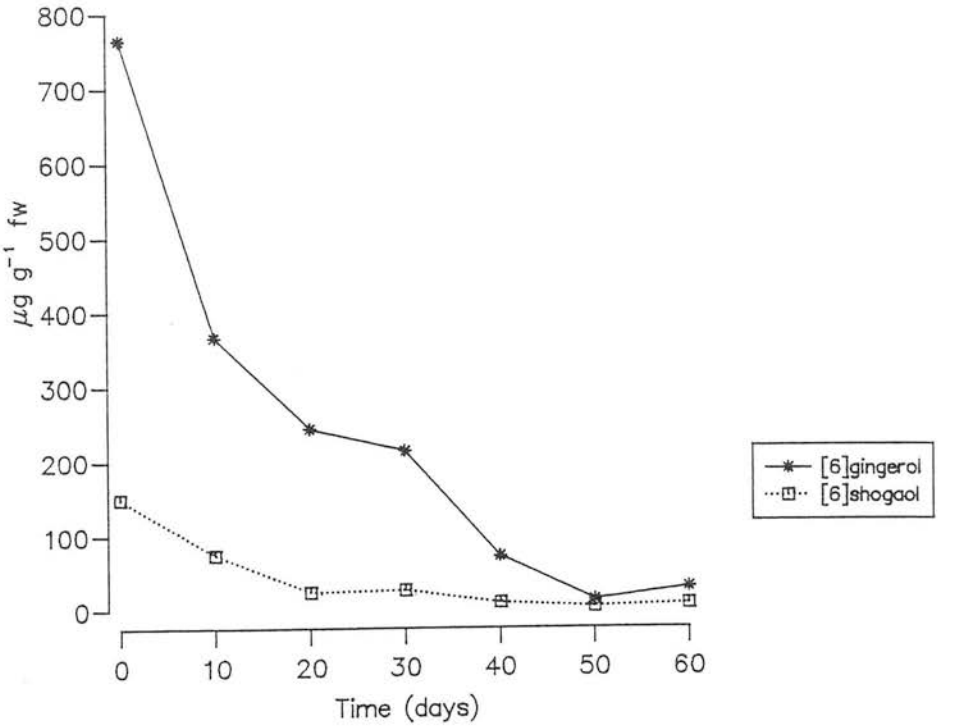
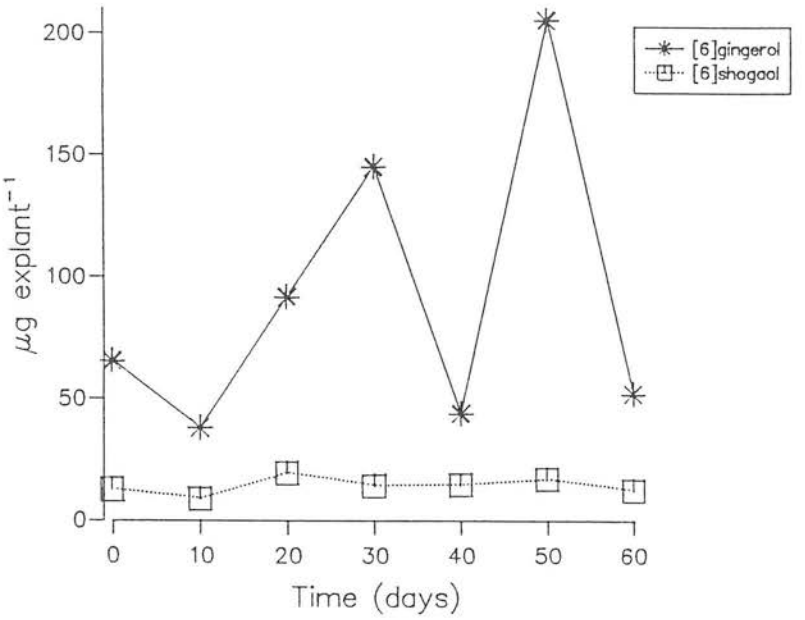


Figure 3.5.3c-d Amount (per explant) of [6]gingerol and [6]shogaol in explants cultured on **(c)** 1/2MSB medium and **(d)** SHB medium. Each value is the mean of three replicates

(c)



(d)

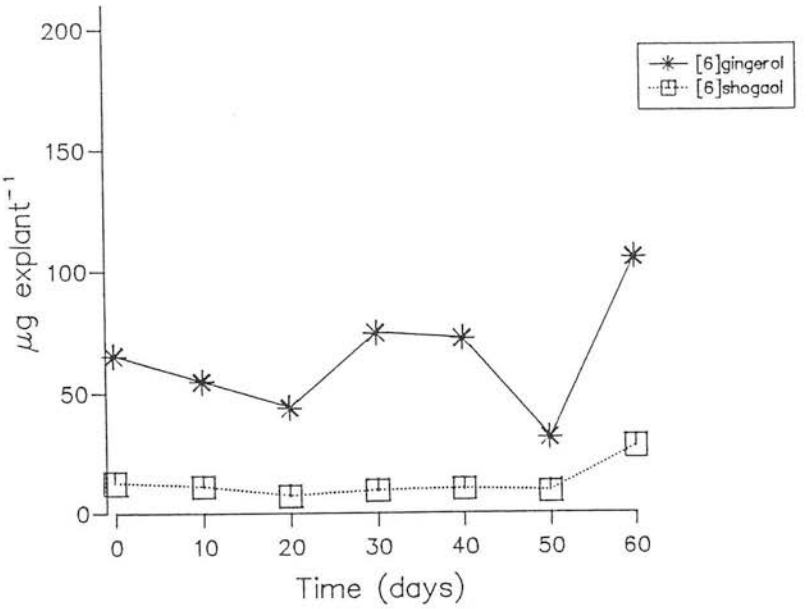
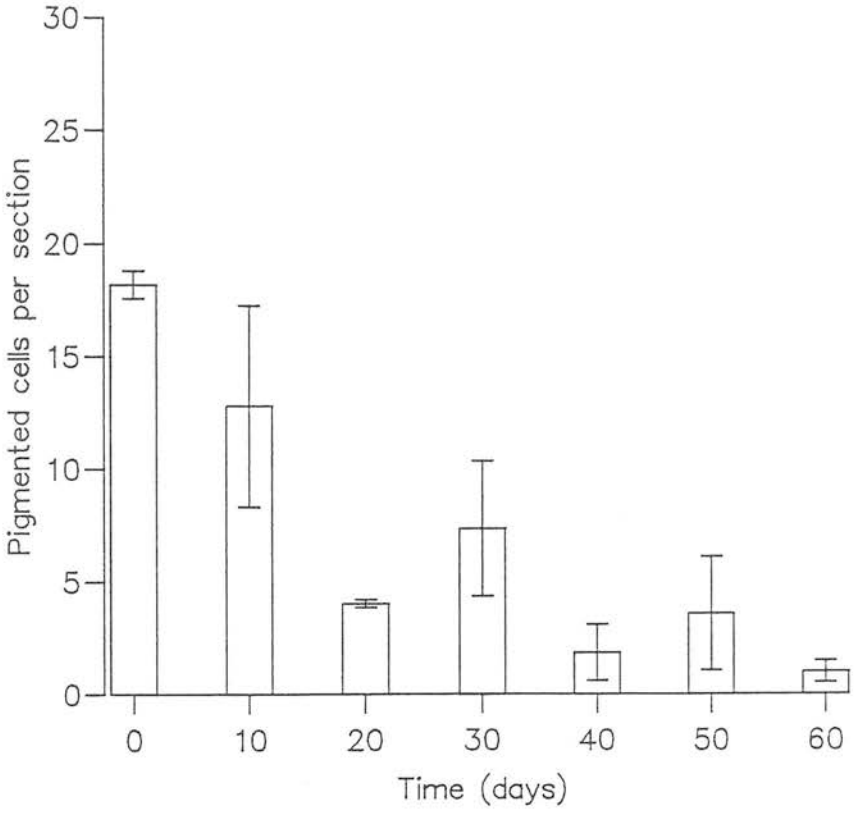
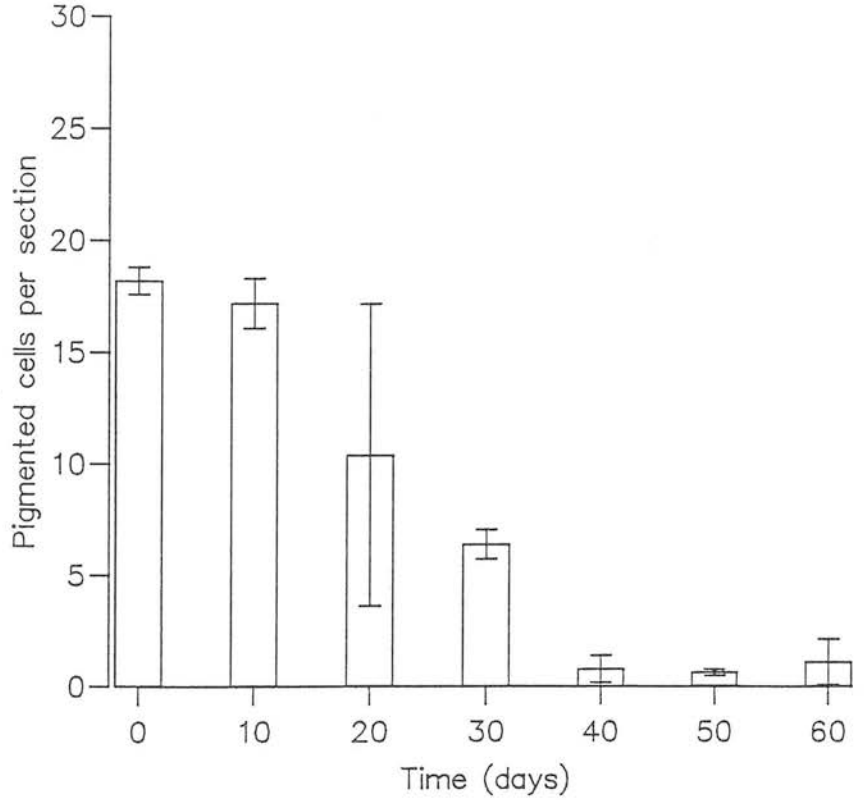


Figure 3.5.4a-b Number of yellow pigmented cells per section of explants cultured on **(a)** 1/2MSB medium and **(b)** SHB medium. Each value is the mean of three replicates ± se

(a)



(b)



The results illustrated in Figs. 3.5.4a-b show the number of yellow pigmented cells per section in tissue explants which decreased with time from a maximum value at the beginning of culture, reaching to a minimum value at d 40-60 when the cultures show the highest fw and protein content, this coincided with a decrease in tissue organisation as the mass of callus increased. Therefore these results might indicate that despite an increase in tissue disorganisation as callus developed and considering the rise of [6]gingerol (particularly on 1/2MSB) and the decrease in the number of yellow cells it would appear that the remaining coloured cells could contain higher amount of this compound. The lower proportion of pigmented cells may be due to an increase in the number of non-pigmented cells in the explants.

In order to assess the differences in the number of pigmented cells recorded between the two treatments and to decide which of the culture media was more favourable for the appearance of these cells a *t*-test was performed for each sampling time. Results show no significant difference in the number of pigmented cells (at P=0.05) between explants cultured on the two different media at any of the sampling intervals (see Appendix).

In the next sub-section a similar approach was used but regenerating cultures were employed. This enabled a comparison to be made between the accumulation of PPPs and morphological differentiation.

3.5.2 Characterisation of growth and accumulation of phenolic pungent principles in regenerating explants of *Z.officinale*

There are a number of reports in the literature which suggest that morphological as well as biochemical differentiation favour the synthesis and accumulation of a number of secondary metabolites in culture (Yeoman *et al.*, 1980; Ozeki and Komamine, 1981; Yeoman *et al.*, 1982; Holden *et al.*, 1987; Flores *et al.* 1987 and Komamine *et al.* 1989). In the following experiments an attempt was made to show

whether the accumulation of PPPs in ginger was affected by the induction of morphological differentiation.

Plant regeneration was achieved with two solid growth media MS 5/1 and MS 3/0.5 (see Table 3.4.4). Previously it was reported in this thesis (3.4.2) that the extent of plantlet formation from rhizome explants was higher on MS 3/0.5 than on MS 5/1 (see Table 3.4.5). In this experiment the growth and accumulation of PPPs was studied from regenerated ginger plants on two culture media MS 5/1 and MS 3/0.5 over a culture period of 50d (see Table 3.4.4) under the conditions stated in 2.2.3.1.

Growth was followed by measuring changes in fw and protein content (see 2.2.4.1 and 2.2.4.5). Histochemical analysis and counting of the yellow pigmented cells was performed on rectangular pieces of tissue 2.5x2.0mm, with a fw of *ca.* 0.02g taken from explants frozen and then sectioned at a thickness of 25-30μm using a cryostat (2.7.1). The yellow pigmented cells were counted under a light microscope after the addition of a 10% (w/v) solution of sodium carbonate (2.7.4). The replicates for each sample time were bulked up for extraction of the PPPs in acetone (2.3.2.1); quantitative analysis of the extracts was conducted by HPLC (see 2.6). Finally after acetone extraction, the remaining dry material was subjected to protein extraction with 0.1N NaOH and the protein content determined using a BSA calibration curve (2.2.4.5).

Results presented in Figs. 3.5.5a-b show the changes in fw of explants cultured on both MS 5/1 and MS 3/0.5. It can be seen that on both media the explants have undergone an increase in fw immediately after culture initiation with no apparent lag phase. The fw recorded at d 20 show increases of 12.9 fold and 9.1 fold for MS 5/1 and MS 3/0.5 respectively with no significant difference between treatments (at P=0.05) (see Appendix) After d 20 no further increase in fw was recorded for explants on MS 5/1. After d 30 the fw decreased until the end of the culture period. It was also observed that the number of regenerated plants was lower on this medium

with a much lower increase in fw compared to that on MS 3/0.5 (see Table 3.4.5). Explants cultured on MS 3/0.5 showed no further increase from d 30 onwards. At d 50 there was an overall increase in fw of 29.8 fold which appears to be significantly different to that achieved with MS 5/1 cultures (at P=0.05) (see Appendix). Similarly the results presented in Figs. 3.5.6a-b show the changes in protein content of explants cultured on MS 5/1 and MS 3/0.5. From these results it can be seen that protein content follows a similar pattern to the changes reported for fw (see Figs. 3.5.5a-b). In the case of explants cultured on MS 5/1 there is an increase up to d 20 of 13 fold followed by a period until d 40 with no increase in protein level which eventually decreased markedly by d 50. Alternatively, explants cultured on MS 3/0.5 show a sharp increase in protein content from the onset of culture until d 30 of 24 fold followed by a period without a further significant increase until the end of the experiment.

The extent of induction of morphologically differentiated structures was also different between treatments with MS 3/0.5 showing more plantlets than MS 5/1 this parallels the different fw and protein content recorded for these treatments (see Table 3.4.5).

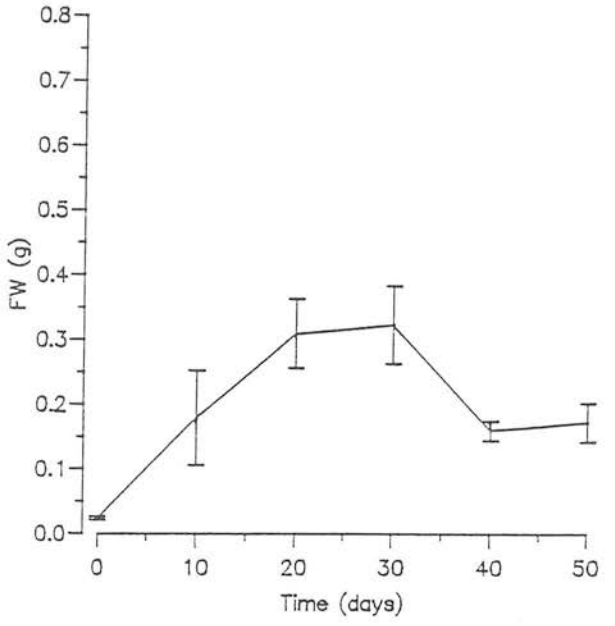
Table 3.4.5 Effect of culture medium on the induction of morphogenetic structures and plantlets from explants of *Z.officinale* after 40d

	Medium	
	MS 5/1 medium	**MS 3/0.5 medium**
Contamination	28.0% **a**	24.0% **a**
Root induction	26.6% **a**	13.3% **a**
Shoot induction	26.6% **a**	26.6% **a**
Plantlet induction	6.60% **a**	33.3% **b**
Callus formation	40.0% **a**	26.6% **a**

Values within a row not followed by the same letter differ significantly (P=0.05) by X^2-test analysis (df 1)

Figure 3.5.5a-b Changes in the fw of explants cultured on **(a)** MS 5/1 medium and **(b)** MS 3/0.5 medium. Each value is the mean of three replicates ± se

(a)



(b)

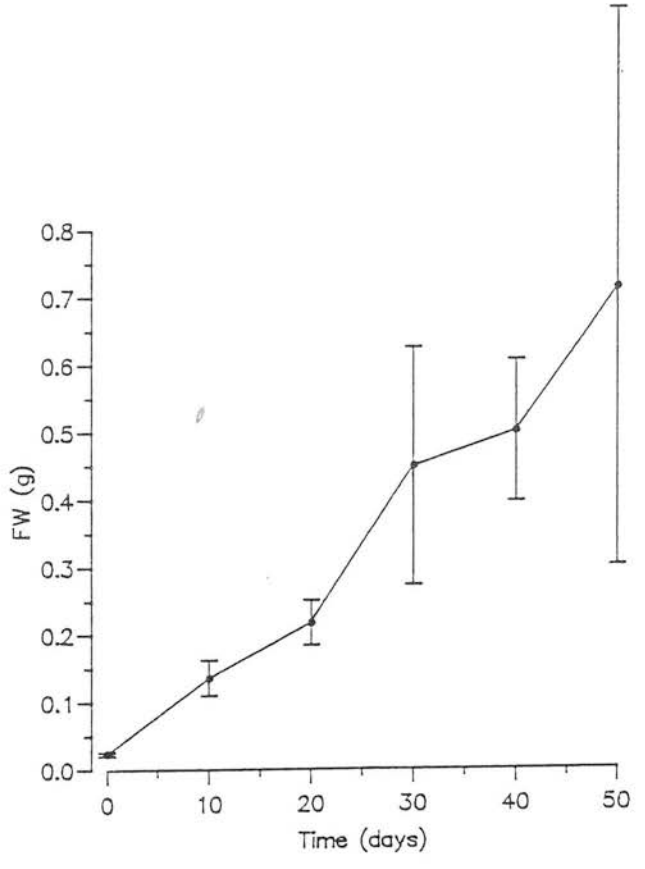
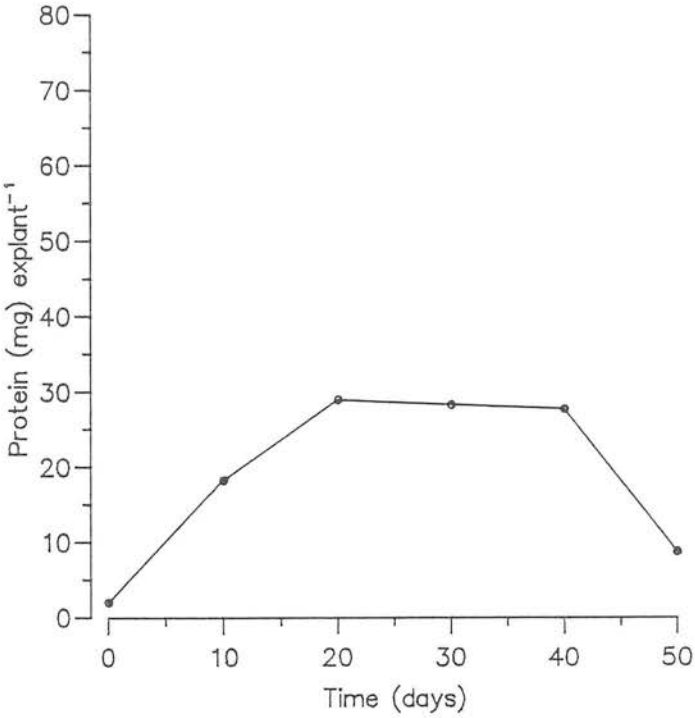
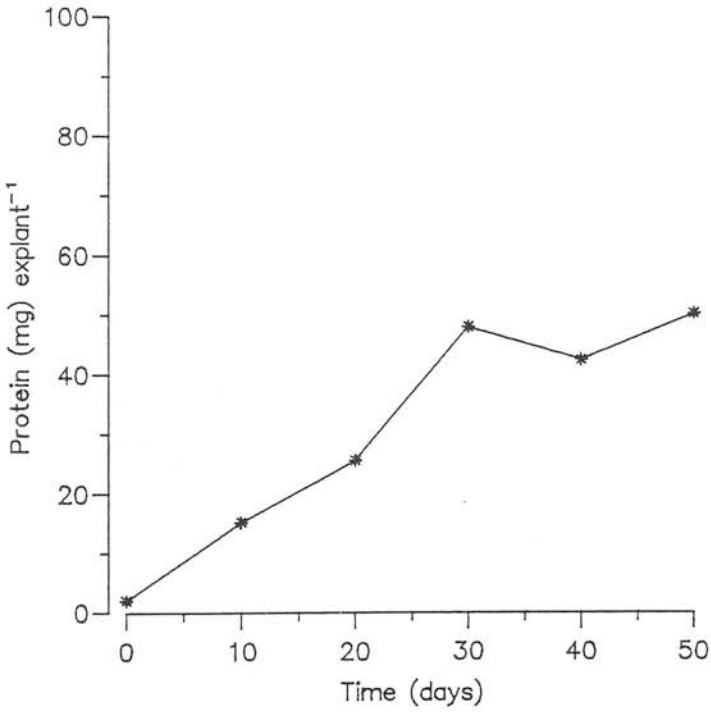


Figure 3.5.6a-b Changes in protein content of explants cultured on **(a)** MS 5/1 medium and **(b)** MS 3/0.5 medium. Each value is the mean of three replicates

(a)



(b)



Results of the quantitative analysis of the acetone extract per g fw of explants cultured on the two media tested are presented in Figs. 3.5.7a-b. These show a sharp decrease in both [6]gingerol and [6]shogaol in explants cultured on both media and this was evident within the first 10d in culture. Thereafter there was no further decrease in these compounds. The results presented in Figs. 3.5.7c-d show the changes in the amount per explant of [6]gingerol and [6]shogaol in explants cultured on two regenerating media. It can be seen that the amount of [6]shogaol remained almost constant on the two media. However, the amount of [6]gingerol in explants cultured on MS 5/1 (see Fig. 3.5.7c) increased from the onset of culture until d 20 showing an overall increase of 2.8 fold. After d 20 the amount of this compound decreased reaching the lowest value at d 40. It was observed that the changes in the amount of [6]gingerol followed a similar trend to the changes in fw (see Fig. 3.5.5a) although the overall increase was very much lower also when the fw declined the amounts of [6]gingerol also dropped. The presence of PPPs in the solidified medium was not assessed due to the difficulty in the extraction, accordingly the possibility of PPPs present in this fraction could not be left out. It would appear that in this culture system the accumulation of [6]gingerol was related to growth and possibly to the increase in morphological differentiation but did not keep pace with growth.

The results presented in Fig. 3.5.7d show the changes per explants of [6]gingerol in explants cultured on MS 3/0.5. It can be seen that after a slight drop at d 20 the amount of [6]gingerol rose sharply with an overall increase of 4.0 fold at d 50. It would appear contrarily to the results obtained with explants cultured on MS 5/1 that the increase in the amount of [6]gingerol did not parallel growth (see Fig. 3.5.5b).

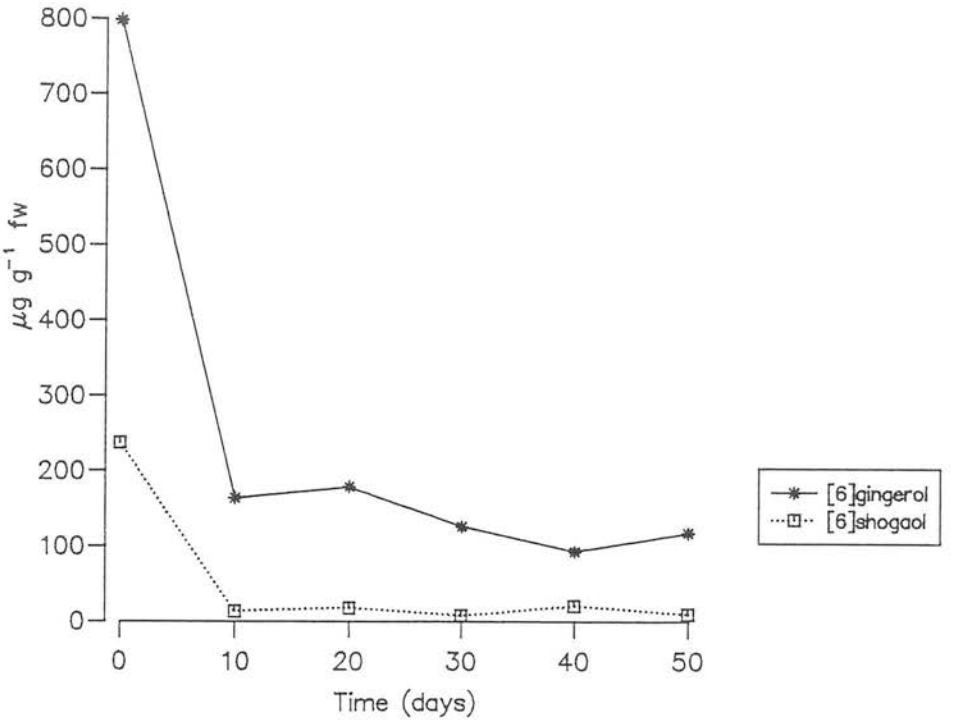
Results for the numbers of yellow pigmented cells presented in Figs. 3.5.8a-b show that the highest number of coloured cells per section was present at the onset of culture. Furthermore, the number of pigmented cells declined gradually throughout the experimental period reaching a minimum value at the completion of the

experiment (40-50d), a similar response to that obtained from explants cultured on callus inducing media (see 3.5.1).

Statistical analysis (P=0.05) shows no significant difference between the number of pigmented cells per section in explants cultured on the two media at any of the sampling intervals (see Appendix). It was decided to compare these results with those achieved during callus induction experiments (see 3.5.1) to assess differences between the number of coloured cells in induced callus and explants displaying plant regeneration. It was observed that a higher number of coloured cells was found on d 10 and 20 in explants from callus but statistical difference was only observed at d 10 between SHB and MS 5/1 and MS 3/0.5 (at P=0.05); similarly the differences in the number of these cells at further intervals was not significant either except at d 30 between 1/2MSB and MS 3/0.5 (see Appendix). Therefore it would appear that despite the increase in tissue organisation during plant regeneration this did not result in greater numbers of pigmented cells per section than during callus induction although a clear increase in [6]gingerol accumulation was observed particularly in explants cultured on regenerating medium MS 3/0.5.

Figure 3.5.7 a-b Amount (per g fw) of [6]gingerol and [6]shogaol in explants cultured on **(a)** MS 5/1 medium and **(b)** MS 3/0.5 medium. Each value is the mean of three replicates

(a)



(b)

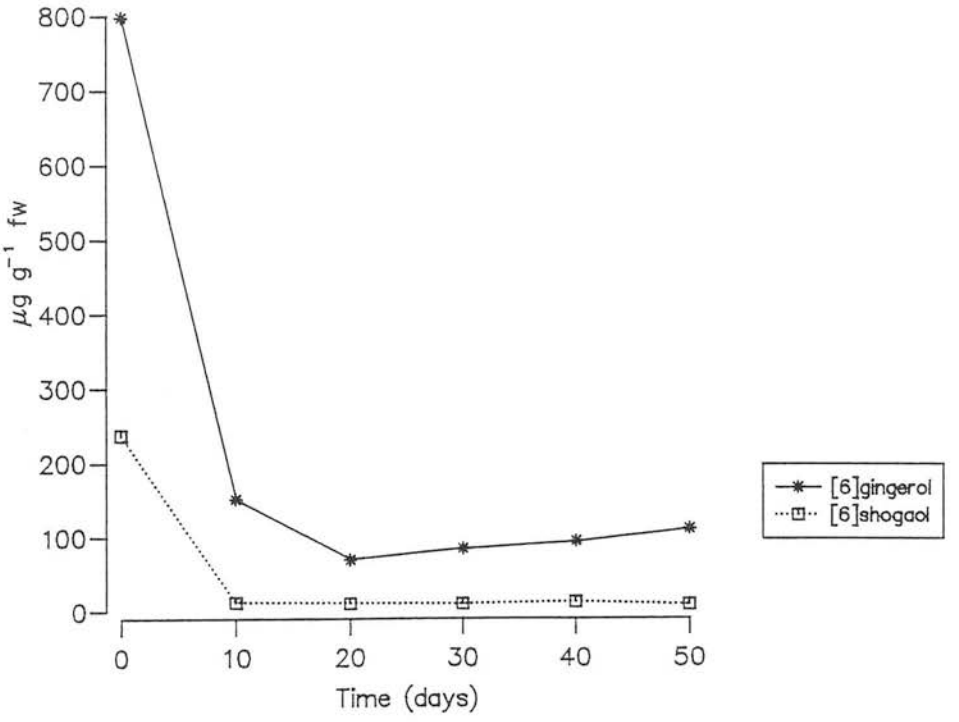
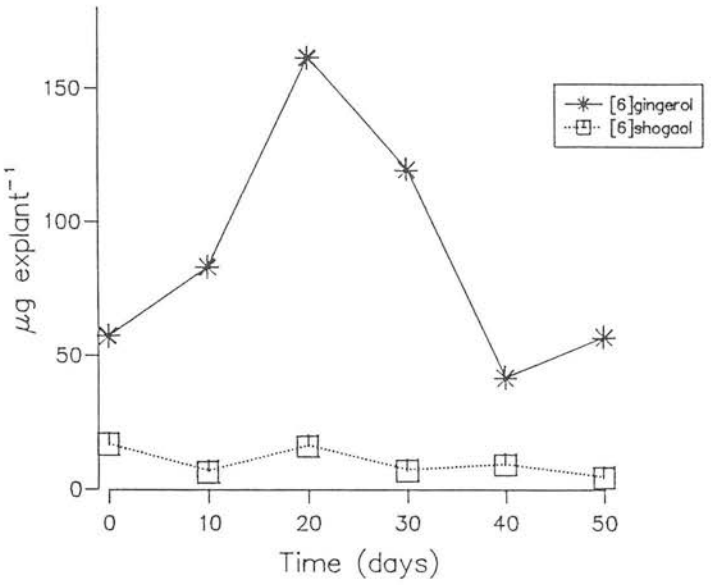


Figure 3.5.7c-d Amount (per explant) of [6]gingerol and [6]shogaol in explants cultured on **(c)** MS 5/1 medium and **(d)** MS 3/0.5 medium. Each value is the mean of three replicates

(c)



(d)

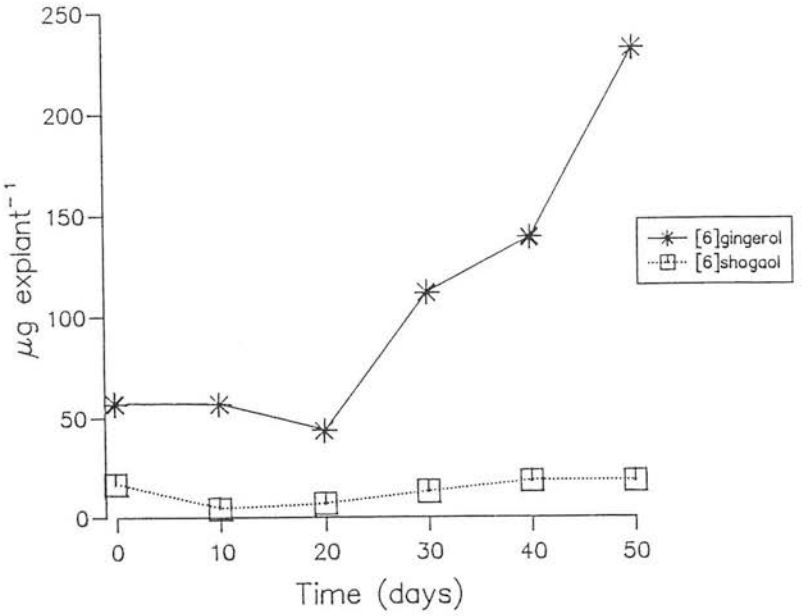
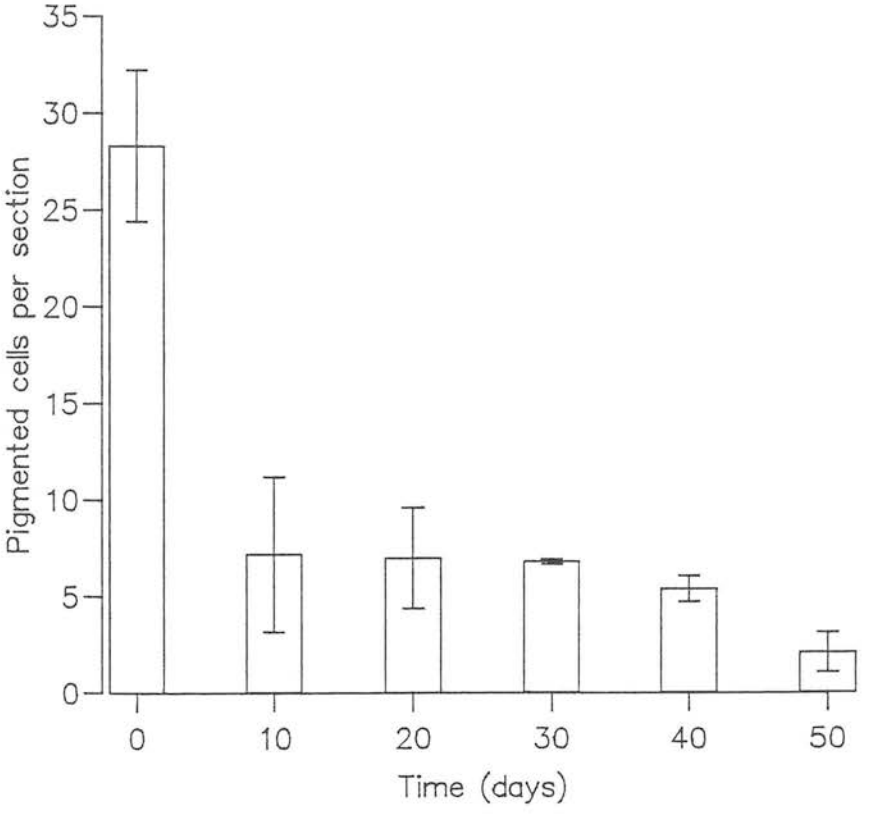
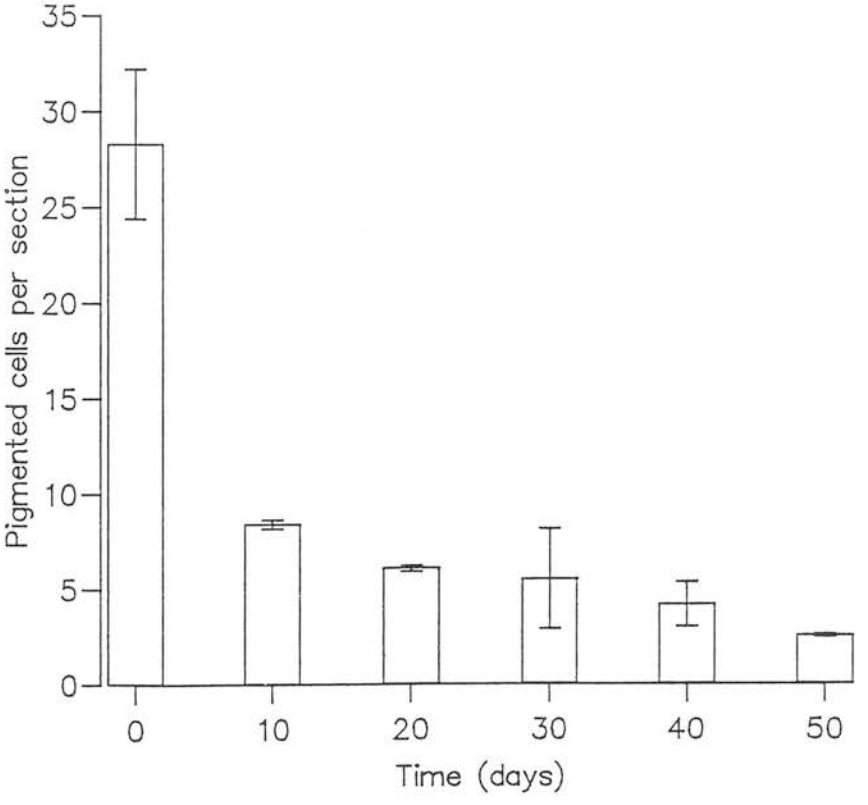


Figure 3.5.8 a-b Number of yellow pigmented cells per section in explants cultured on **(a)** MS 5/1 medium and **(b)** MS 3/0.5 medium. Each value is the mean of three replicates ± se

(a)



(b)



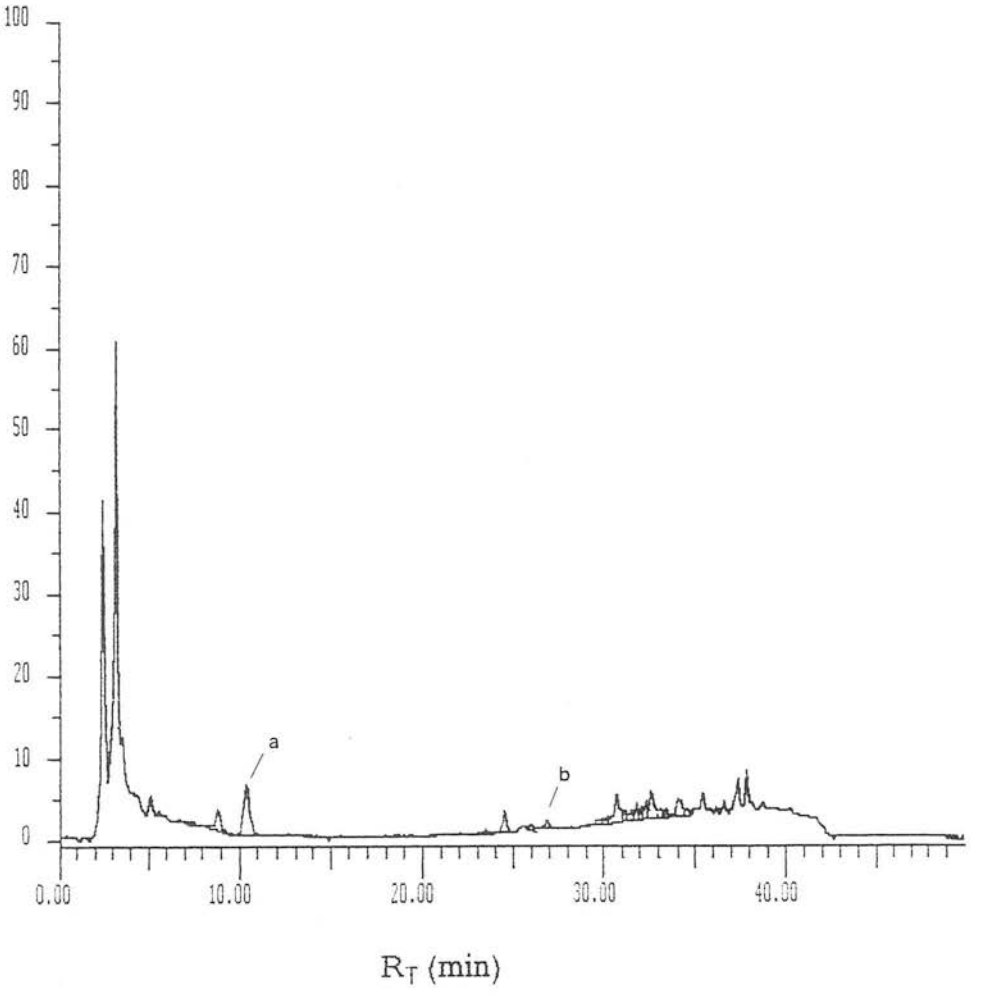
Subsequently, it was decided to study the appearance of these metabolites in more mature plants. Accordingly the remaining plantlets from explants cultured on MS 5/1 and MS 3/0.5 were transferred individually onto 15ml of a solid growth medium of a different composition to the regeneration medium consisting of MS, 3% sucrose, and no PGRs. This solid medium was contained in glass jars (6cm diameter, 11.5cm height) sealed with a small Petri dish, and double sealed with Parafilm. Plantlets cultured under standard conditions (2.2.3.1) developed into plants bearing minute rhizomes (see Fig. 3.5.9a-b) which were then separated and extracted with acetone as described in 2.3.1. Quantitative analysis of acetone extracts of the mini-rhizomes by HPLC (2.6) showed the presence of [6]gingerol and [6]shogaol together with other phenolics (see Fig. 3.5.9c).

Therefore it would appear that the amount of [6]gingerol per explant increased in both culture systems (callus and regenerating inducing media) but this was more evident in the regenerating medium MS 3/0.5. Furthermore, the number of yellow cells per section was observed to decline in both instances (callus and regenerating media) although the number of non-pigmented cells has increased which may have change the proportion in the number of yellow cells which perhaps were not formed. Furthermore, bearing in mind that cell counting of pigmented cells was not performed for the entire tissue, it may be possible that the number of yellow cells would vary and this would enable a clear comparison of the response obtained in the entire explant. Moreover, it would also appear that biochemical differentiation had occurred in callus inducing medium resulting in the accumulation of [6]gingerol; conversely, morphological differentiation induced in the regenerating media, manifested in the formation of shoots, roots, or plantlets, may have favour the higher accumulation of [6]gingerol recorded in MS 3/0.5 medium.

Figure 3.5.9 a-b Photographs showing the appearance of a 2 month old plantlet regenerated from an explant cultured on MS 3/0.5 medium. Observe the presence of an emerging shoot and the growth of leaves and root. The newly originated mini-rhizome can also be seen (arrowed)

(a) **(b)**

Figure 3.5.9c HPLC chromatogram of an acetone extract from a mini-rhizome showing the presence of **(a)** [6]gingerol with a R_T of 10.27min, **(b)** [6]shogaol with a R_T of 26.86min



In the next sub-section the pattern of growth and accumulation of PPPs in suspension cultures of *Z.officinale* are investigated.

3.5.3 Characterisation of growth and accumulation of the phenolic pungent principles in suspension cultures of *Z.officinale*

In 3.4.3 it has already been shown that sustained growth of suspension cultures can be achieved in 1/2MSB medium. However, the rate of increase in biomass was very slow only reaching maximum value of 3.5-4g fw over a culture period of 50-60d. It was also observed that the pH of the medium fluctuated considerably during the culture cycle and this possibly affected growth.

The aim of the following experiments was to determine the pattern of growth and accumulation of PPPs in a number of media and to discover whether the addition of glutamine stabilised the pH and improved growth.

Cultures were initiated by inoculating 0.5g aliquots wet weight of suspended cells, which had been filtered through a 64μm sterile nylon mesh, into 40ml of 1/2MSB liquid growth medium contained in 250ml Erlenmeyer flasks. The inoculated flasks were then placed under standard culture conditions (2.2.3.4). Three replicates were taken every 6d over an experimental period of 60d. Determination of fw, PCV and pH (2.2.4) were performed on each flask and the PPPs were extracted both from filtered cells using acetone (2.3.2.2.) and from the liquid growth medium with a 1:1 (v/v) mixture of Et_2O-EtOAc (2.3.2.2. 1). Quantitative analysis of the acetone and Et_2O-EtOAc extracts were carried out by HPLC (2.6) in order to determine the amounts of PPPs present.

Results presented in Figs. 3.5.10a-b show the changes in fw and PCV throughout a culture period of 60d. Both parameters show a small increase over the first 6d followed by a sustained increase in fw (4.8 fold) and in PCV until d 30-36 (4.4 fold). The cultures then stopped growing (stationary phase) probably due to a depletion in nutrients. The pH of the medium was variable reaching a low value of *ca.* 4.3-4.5 over the period d 6 to d 30. After growth had stopped at d 30 the pH rose sharply to values in excess of 7.0 (see Fig. 3.5.10c) suggesting the release of basic compounds

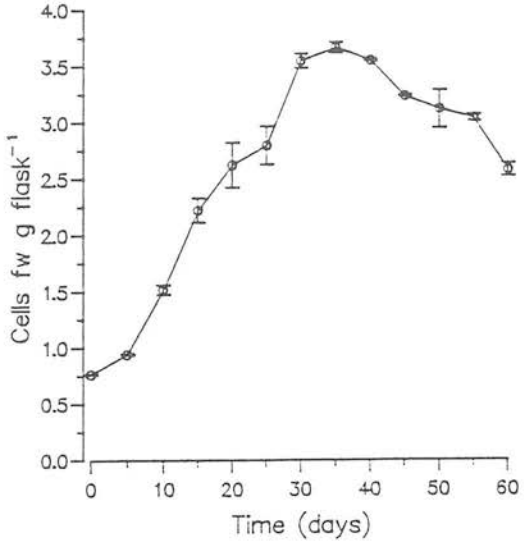
into the medium or a change in the balance between nitrogen forms (ammonium-nitrates) (Veliky and Rose, 1973).

Quantitative analysis of the acetone cell extracts of suspended cells cultured in 1/2MSB showed the presence of vanishingly small amounts of [6]gingerol with values close to zero (see Fig. 3.5.10d). However, quantitative analysis of the Et_2O-EtOAc extracts from the liquid medium (see Fig. 3.5.10e) show the presence of [6]gingerol from d 24 onwards when the culture was still increasing in fw (see Fig. 3.5.10a) a high value of *ca.* 20μg $flask^{-1}$ occurred at d 46 when growth had ceased (see Fig. 3.5.10e). The relatively high amount of [6]gingerol present in the medium which coincided with the cessation of growth could indicate that a particular stage of cellular differentiation has been reached enabling the cells to synthesise and release this compound. This inverse relationship between growth and accumulation of a secondary metabolite is similar to the pattern of secondary metabolite accumulation often reported in the literature *eg.* (Yeoman *et al.*, 1980; Lindsey and Yeoman, 1985; Böhm *et al.*, 1991).

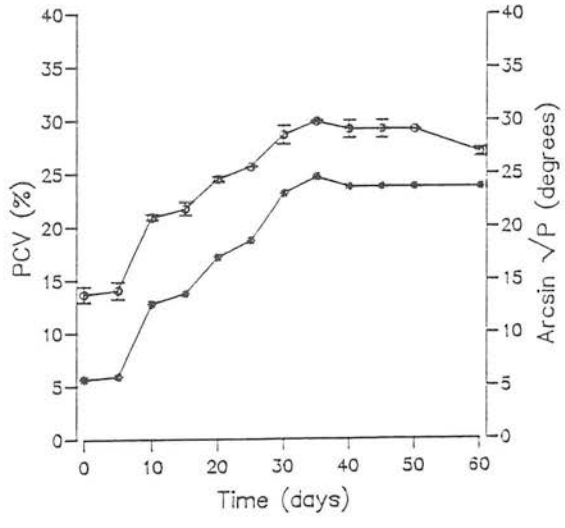
Having observed the changes in pH, attempts were made employing different media to establish a culture medium in which the pH remained constant as this should enhance growth and may alter the yield of PPPs. There are several instances reported in the literature where controlling the pH of the medium increased secondary product yield and differentiation *eg.* tryptophol formation by cultured *Ipomoea* sp. cells (Veliky, 1977); and the induction of carrot somatic embryogenesis (Smith and Krikorian, 1990). It is also generally accepted that the addition of glutamine stabilises the pH.

Figures 3.5.10a-c Changes in **(a)** fw, **(b)** PCV and **(c)** pH of suspended cells cultured in 1/2MSB. (*) Each value is the mean of three replicates, (○) each value is the mean of three replicates ± se after Arcsin transformation (where appropriate)

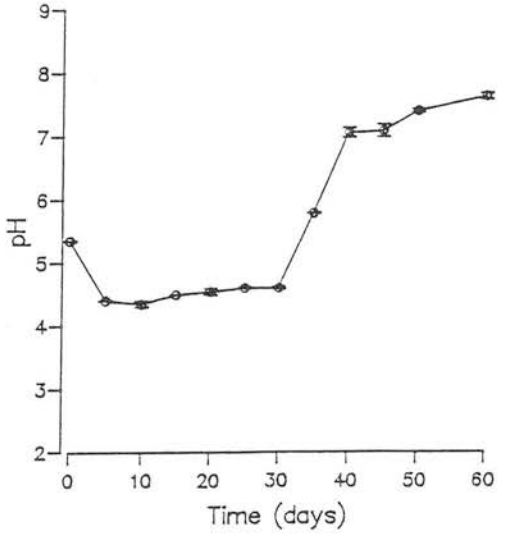
(a)



(b)

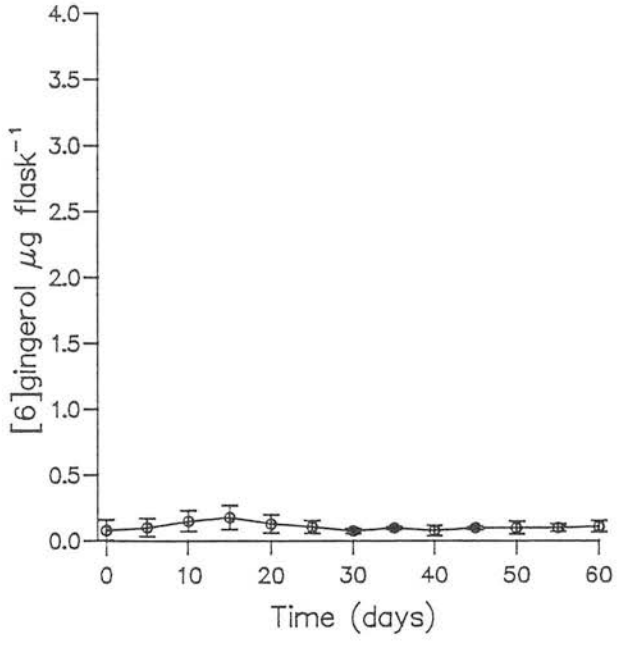


(c)

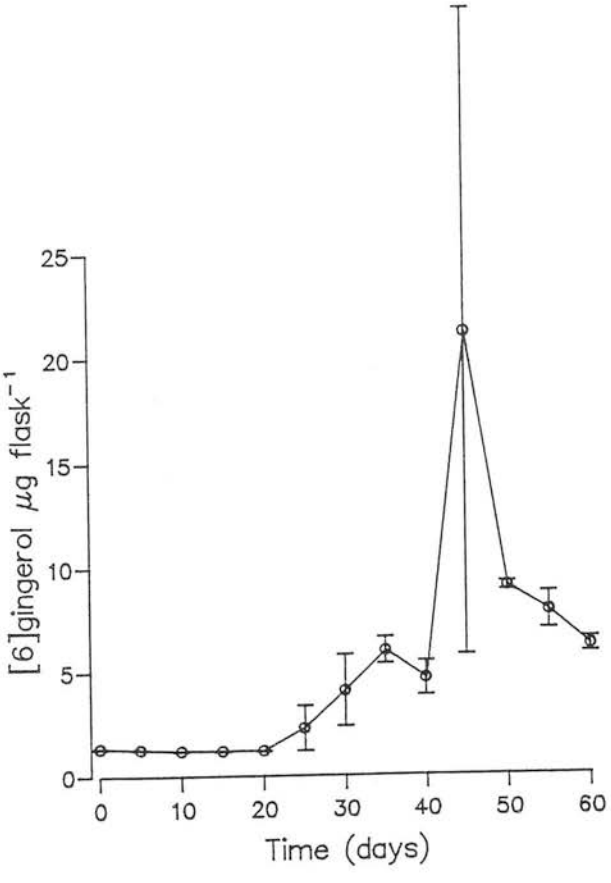


Figures 3.5.10d-e [6]gingerol content of **(d)** acetone cell extracts and **(e)** Et_2O-EtOAc medium extracts of suspension cultures in 1/2MSB medium. Each value is the mean of three replicates ± se

(d)



(e)



Initially 1/2MSBG medium was employed this was 1/2MSB medium with the addition of 100mg l^{-1} glutamine (see Table 3.5.1) In further experiments SHB and SHBG, SHB medium supplemented with 100mg l^{-1} glutamine, were also employed (see Tables 3.4.3 and 3.5.1).

Growth parameters fw, and PCV together with pH and PPP content, both from cells and medium were measured in three replicates taken every 6d over an experimental period of 60d. Initiation and culture maintenance were as described above.

Results presented in Figs 3.5.11a-b show the changes in fw and PCV of suspended cells cultured in 1/2MSBG medium. It can be seen that after a small increase over *ca.* 6d (note similar behaviour to suspended cells cultured in 1/2MSB see Fig. 3.5.10a-b), there was a continuous increase in fw and PCV which lasted for *ca.* 25d achieving a maximum fw value of 3.35g flask^{-1}. Subsequently, cultures ceased growth and entered a stationary phase. Results presented in Fig. 3.5.11c show that the pH was variable as in suspended cells cultured in 1/2MSB (see Fig. 3.5.10c). Here the pH dropped to values of *ca.* 4.0 at d 6 and remained unchanged until d 20. After d 20 the pH rose to *ca.* 7.4, which coincided with the onset of the stationary phase. From these results it can be concluded that the addition of 100mgl^{-1} glutamine did not produce a stable pH and the results are very similar to those reported for 1/2MSB.

Quantitative analysis of the acetone and Et_2O-EtOAc extracts from cells and medium respectively are presented in Figs. 3.5.11d-e. It can be seen from Fig. 3.5.11d that a very low amount of [6]gingerol is present in the cell extract with values no higher than 0.20μg flask^{-1}. However, results presented in Fig. 3.5.11e show the presence of [6]gingerol in the medium at levels of *ca.* 2.5μg flask^{-1} at d 18 which then increased further reaching values of *ca.* 5.0μg flask^{-1} at d 24 when the cultures had entered the stationary phase (see Fig. 3.5.11a-c). Subsequently, [6]gingerol was absent from the medium for the rest of the experimental period. No other related PPPs

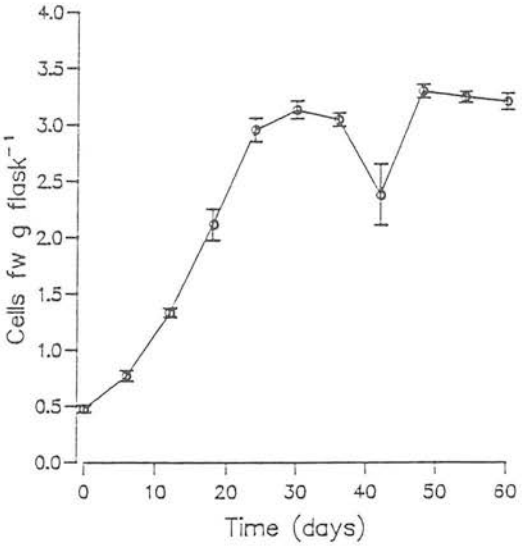
were detected. There is a significant difference between the amount of [6]gingerol present in the medium in 1/2MSB compared to that recorded in 1/2MSBG (see Figs. 3.5.10e and 3.5.11e) (see Appendix). Therefore, it would appear that the addition of 100mg l^{-1} glutamine alters the pattern of [6]gingerol accumulation resulting in early synthesis and release into the medium which would suggest that the cells reached a stage of biochemical differentiation earlier than that recorded in 1/2MSB. However this accumulation did not last long.

Table 3.5.1 Composition of two liquid growth media, 1/2MSBG and SHBG which are 1/2MSB and SHB respectively supplemented with 100mg l^{-1} glutamine

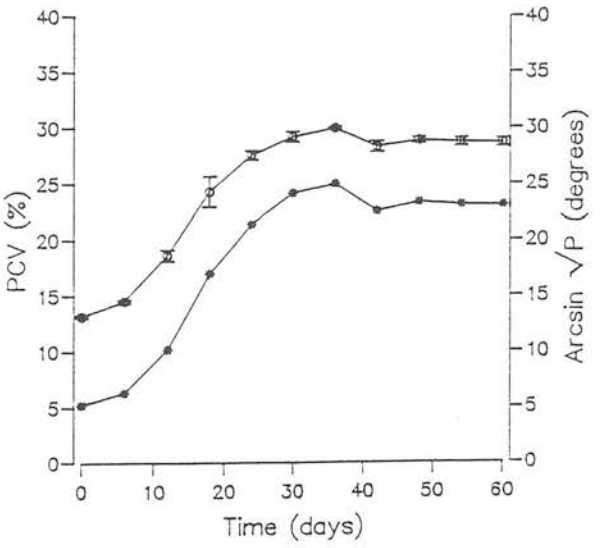
1/2MSBG medium	**SHBG medium**
1/2 MS	SH
3% sucrose	3% sucrose
100mg l^{-1} glutamine	100mg l^{-1} glutamine
1mg l^{-1} 2,4-D	1mg l^{-1} 2,4-D
0.5mg l^{-1} BAP	0.1mg l^{-1} Kin
1mg l^{-1} CPA	
pH 5.8	pH 5.8

Figures 3.5.11 a-c Changes in **(a)** fw, **(b)** PCV and **(c)** medium pH of suspended cells cultured in 1/2MSBG. (*) Each value is the mean of three replicates, (○) each value is the mean of three replicates ± se after Arcsin transformation (where appropriate)

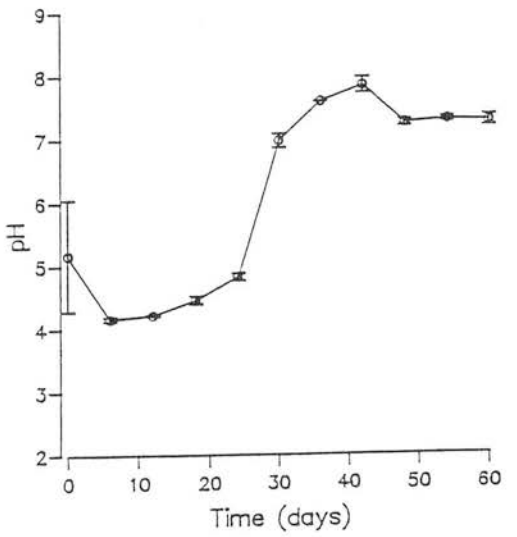
(a)



(b)

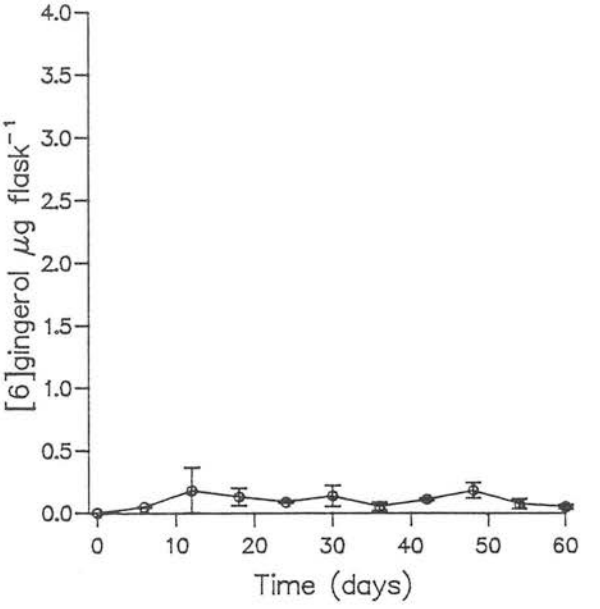


(c)

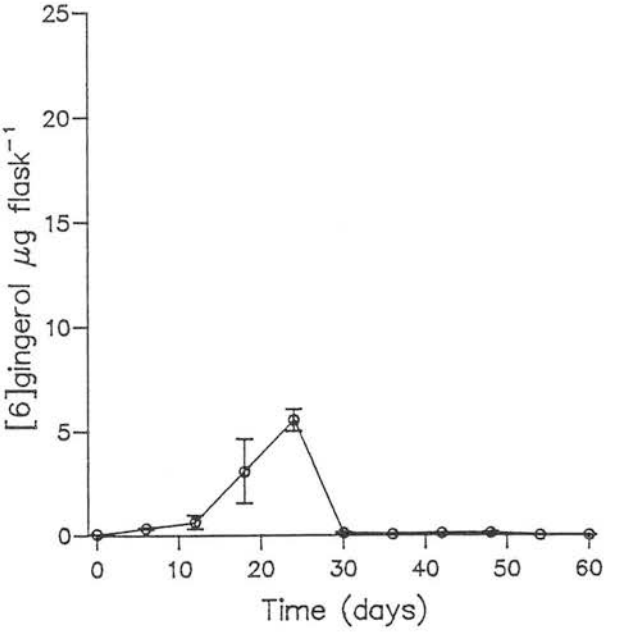


Figures 3.5.11 d-e [6]gingerol content of **(d)** acetone cell extracts and **(e)** Et_2O-EtOAc medium extracts of suspended cells cultured in 1/2MSBG. Each value is the mean of three replicates ± se

(d)



(e)



In order to assess the effect of culture medium on growth and PPP accumulation a similar investigation to that already described was carried out with two other media SHB and SHBG which are based on SH salts and possess a lower level of nitrogen, and differ in the PGRs added (see Tables 3.4.2 and 3.5.1) .

The experiment was set up as described previously by inoculating 0.5g wet weight of filtered cells into 40ml of liquid medium. Three flasks for each treatment were sampled at 6d intervals over a culture period of 60d.

Results presented in Figs. 3.5.12a-b show the changes in fw and PCV of cells cultured in SHB. Unlike cells cultured in 1/2MSB and 1/2MSBG (see Figs 3.5.10a-b and 3.5.11a-b), there was an initial lag phase of approximately 12d, followed by a steady increase after d 30 in both fw (8 fold) and PCV (3.6 fold) respectively. The maximum fw achieved at this time was 3.6g flask^{-1}, and there was no significant difference with the values obtained in 1/2MSB and 1/2MSBG (see Figs. 3.5.10a-b and 3.5.11a-b) (see Appendix). The changes in pH over the culture period are shown in Fig. 3.5.12c. As previously the rapid drop in pH to values close to 4.5 during the lag phase lasted for *ca.* 12d. After this, in contrast to the pattern recorded for 1/2MSB and 1/2MSBG, the pH rose to values of *ca.* 5.8 from d 18 to d 38 which coincided with a steady increase in fw and PCV. This was then followed by a further rise throughout the stationary phase of growth reaching values near to 8.0.

The amounts of the PPPs in cells and medium are presented in Fig. 3.5.12d-e and show the presence of [6]gingerol in the cells at d 18, when cultures appeared to be at the beginning of active growth. The amount of this metabolite increased further reaching a maximum value of 7.3µg flask^{-1} at d 30 which coincided with the onset of the stationary phase. During the stationary phase there was sharp drop in the amount of [6]gingerol suggesting perhaps the cessation of synthesis or an increase in degradation. The results in Fig. 3.5.12e show the amount of [6]gingerol liberated into the medium which is very low and close to the detection limit with a maximum value

of just 0.25µg flask^{-1} at d 36. In both cells and medium the [6]gingerol appeared to be the only PPPs present.

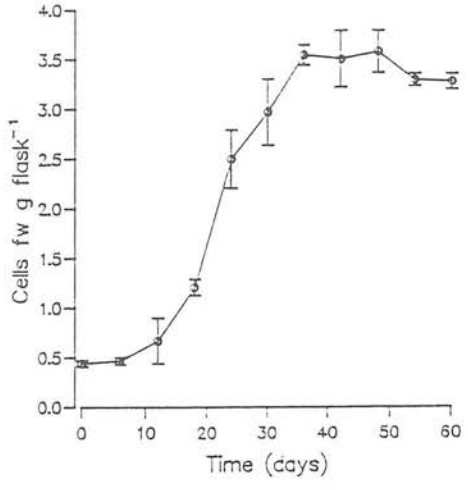
From this it would appear that in this treatment cells do not release this metabolite into the medium unlike the results obtained from 1/2MSB and 1/2MSBG cultures (see Figs. 3.5.10e and 3.5.11e).

The results obtained using SHBG medium, SHB supplemented with 100mgl^{-1} glutamine (see Table 3.5.1) are presented in Figs. 3.5.13a-b and show the changes in fw and PCV of suspended cells. Here there was no initial lag phase (as in 1/2MSB and 1/2MSBG) with a steady increase in both fw and PCV reaching a maximum value at d 30 at which there was an overall increase of 7.8 and 3.6 fold in fw and PCV respectively. The maximum fw (3.5g flask^{-1}) reached at d 30 was not significantly different to the maximum values obtained with the previous three media (1/2MSB, 1/2MSBG and SHB) (see Appendix). At the end of the culture period there was marked reduction in fw which was not paralleled by the PCV. In Fig. 3.5.13c it can be seen that the pH dropped initially to 4.7 and then rose rapidly increasing to *ca.* 6.0 after d 12 and continuing to rise to a maximum value of *ca.* 8.0 by the end of the culture period.

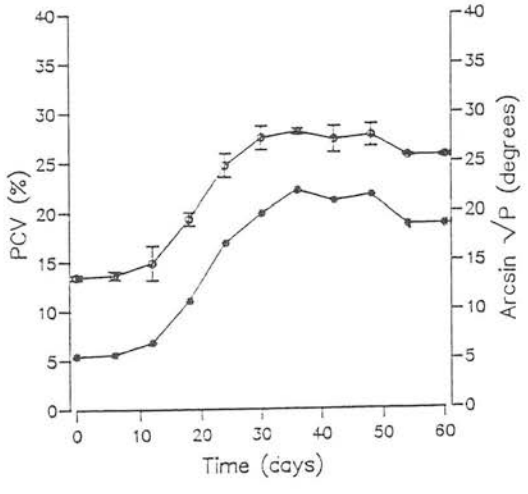
The pHs recorded for these two media SHB and SHBG varied considerably showing maximum values of *ca.* 8.0 by the end of the culture period. From this it would appear that glutamine did not provide a stable pH but does affect the accumulation of these metabolites.

Figure 3.5.12 a-c Changes in **(a)** fresh weight, **(b)** PCV and **(c)** medium pH of suspended cells cultured in SHB. (*) Each value is the mean of three replicates, (○) each value is the mean of three replicates ± se after Arcsin transformation (where appropriate)

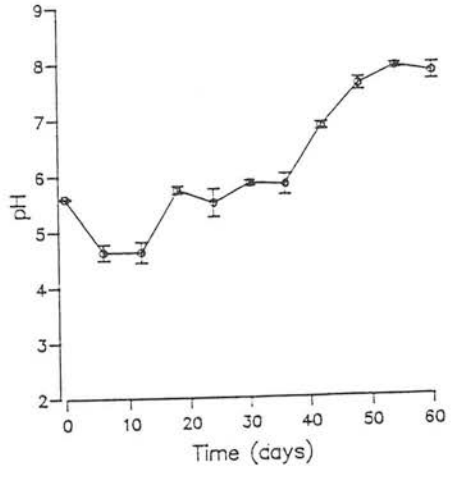
(a)



(b)

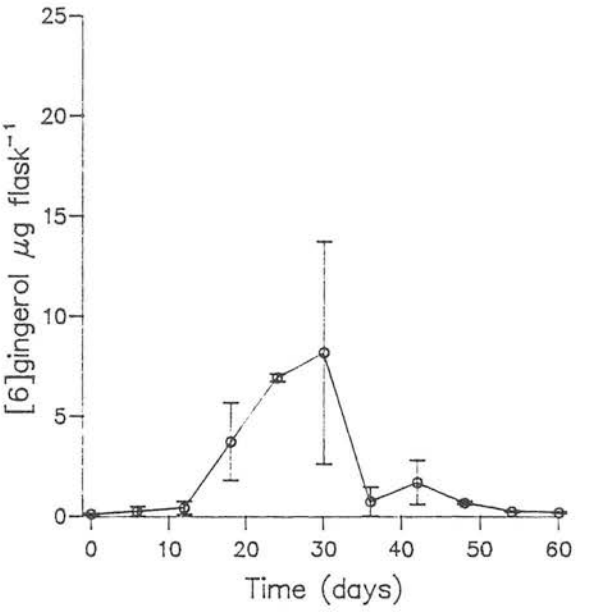


(c)

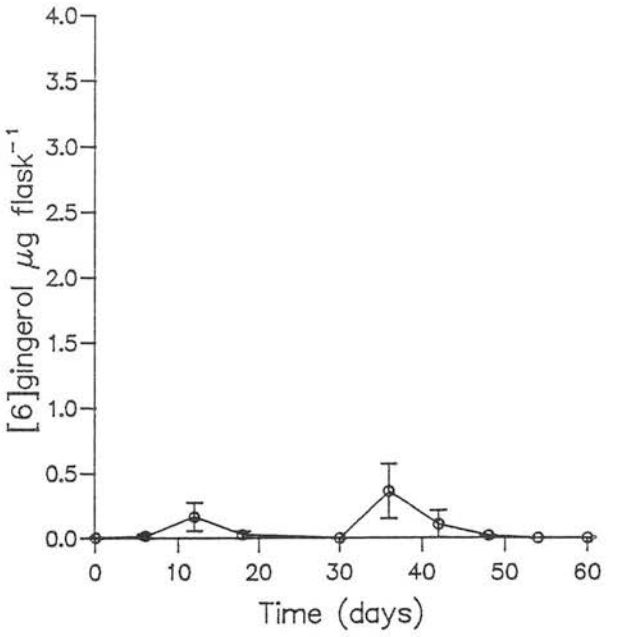


Figures 3.5.12 d-e [6]gingerol content of **(d)** acetone cell extracts **(e)** Et_2O-EtOAc medium extracts of suspended cells cultured in SHB. Each value is the mean of three replicates ± se

(d)

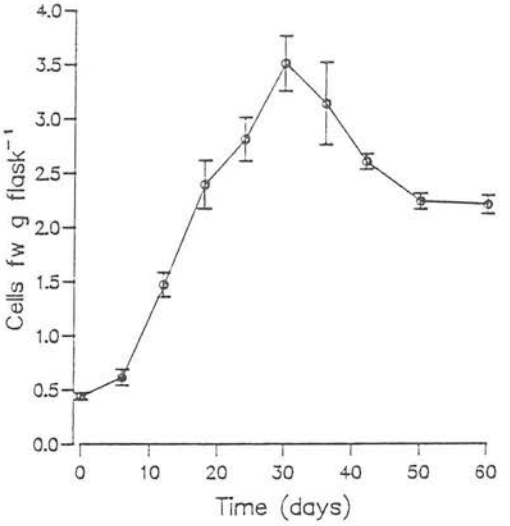


(e)



Figures 3.5.13 a-c Changes in **(a)** fw, **(b)** PCV and **(c)** pH of medium of a suspension culture in SHBG. (*) Each value is the mean of three replicates, (○) each value is the mean of three replicates ± se after Arcsin transformation (where appropriate)

(a)



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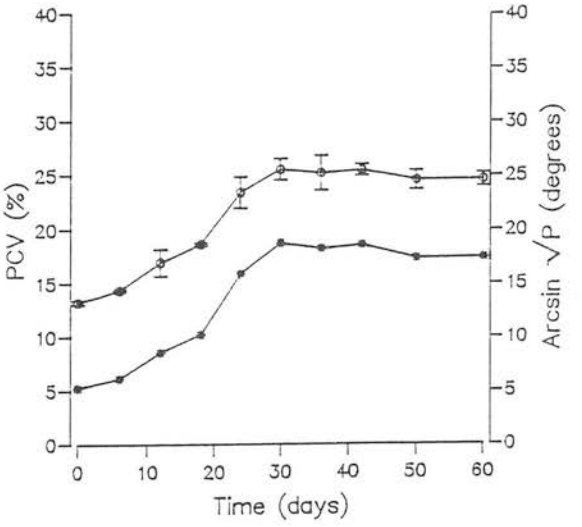
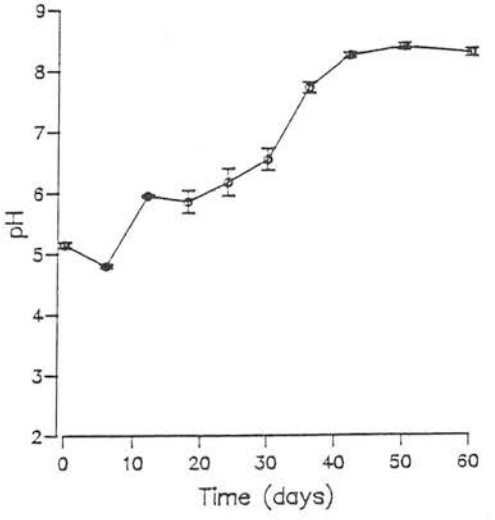
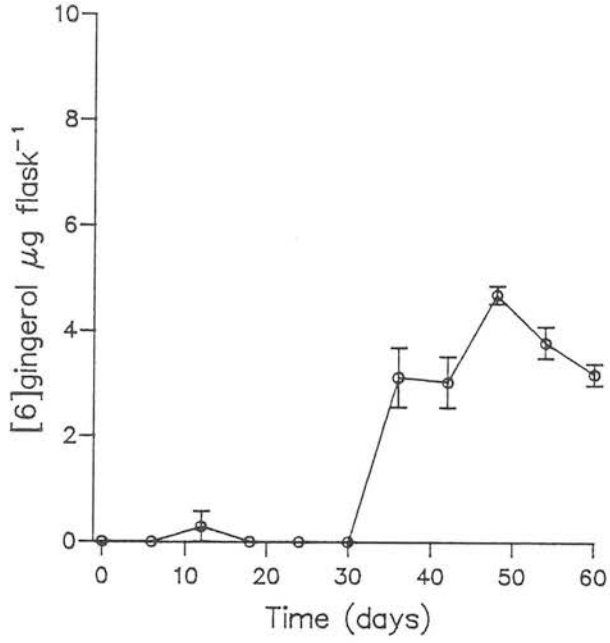


(c)

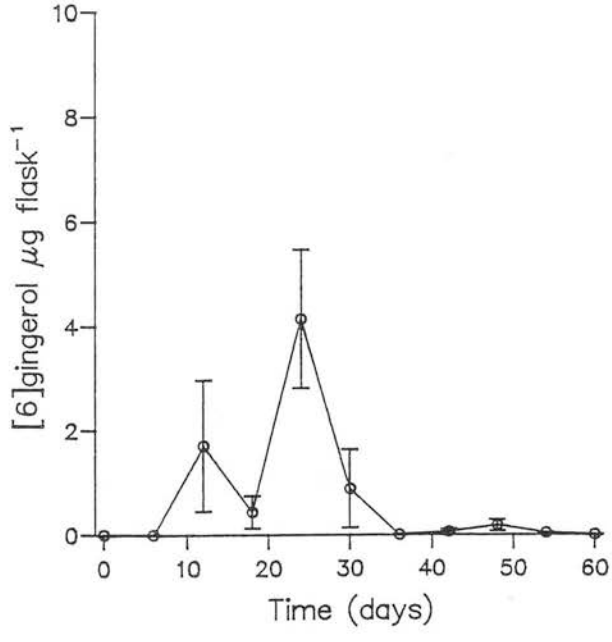


Figures 3.5.13 d-e [6]gingerol content of **(d)** acetone cell extracts **(e)** Et_2O-EtOAc liquid medium extracts of suspended cells cultured in SHBG. Each value is the mean of three replicates ± se

(d)



(e)



The results presented in Fig. 3.5.13d show that very small amount of [6]gingerol was present in the cells at d 12 which drop to zero with a clear increase on d 36 (3μg flask^{-1}). There was a further small increase to reach a maximum amount of *ca.* 4.2μg flask^{-1} by d 48. Subsequently there was a small decrease to *ca.* 3μg flask^{-1}. The results presented in Fig. 3.5.13e show the amount of [6]gingerol present in the medium. This compound was detected at d 12 with values close to 2μg flask^{-1} which remained constant until d 18. A high value was recorded at d 24 when the cultures were approaching their maximum fw value subsequently this compound decreased in amount and had disappeared by the end of the culture period.

Therefore it would appear that the accumulation of [6]gingerol was affected by the composition of the medium as well as by the addition of glutamine.

After establishing that the highest amount of [6]gingerol was reached in cultures in 1/2MSB, and this was mainly released into the medium, it was decided to attempt to increase the yield further by changing the levels of sucrose which has been reported to alter growth and increase the accumulation of secondary metabolites in a number of instances.

3.5.4 Effects of sucrose level upon the growth and accumulation of phenolic pungent principles

It has now been established that suspension cultures grown in media based on MS promote the synthesis and accumulation of [6]gingerol, and this is chiefly released into the medium. Although cultures grown in SH derived media also accumulated [6]gingerol in both cells and medium but to a lesser extent. It was also shown that the addition of glutamine to the media did not prevent a sharp drop in pH, however, it did change the level of phenolics in the cultures (see 3.5.3).

In an attempt to stimulate the accumulation of PPPs further it was decided to stress the cultures by increasing the concentration of sucrose in the medium which is

generally accepted to slow growth, this would also show if the accumulation of the PPPs in cultures is related to growth.

There are a number of references in the literature reporting that higher yields of secondary metabolites can be produced by increasing the concentration of the carbon source, usually sucrose. For example Zenk *et al.* (1977) observed clear increases in alkaloid yield in *Catharanthus roseus* cell cultures when the sucrose concentration was raised above 3% (w/v), a similar observation has been reported by Knobloch and Berlin (1980) with 8% sucrose. Davies (1972) also showed that increasing the sucrose concentration in the medium led to increased polyphenol accumulation in suspension cultures of *Rosa* sp. It has been claimed that some of these effects at the higher sucrose concentrations on growth and metabolism are the results of osmotic stress (Do and Cormier 1990, 1991).

In the following investigation four different media with increasing amounts of sucrose from 3-12% (w/v) were used (see Table 3.5.2) and the effects upon growth and production of PPPs monitored.

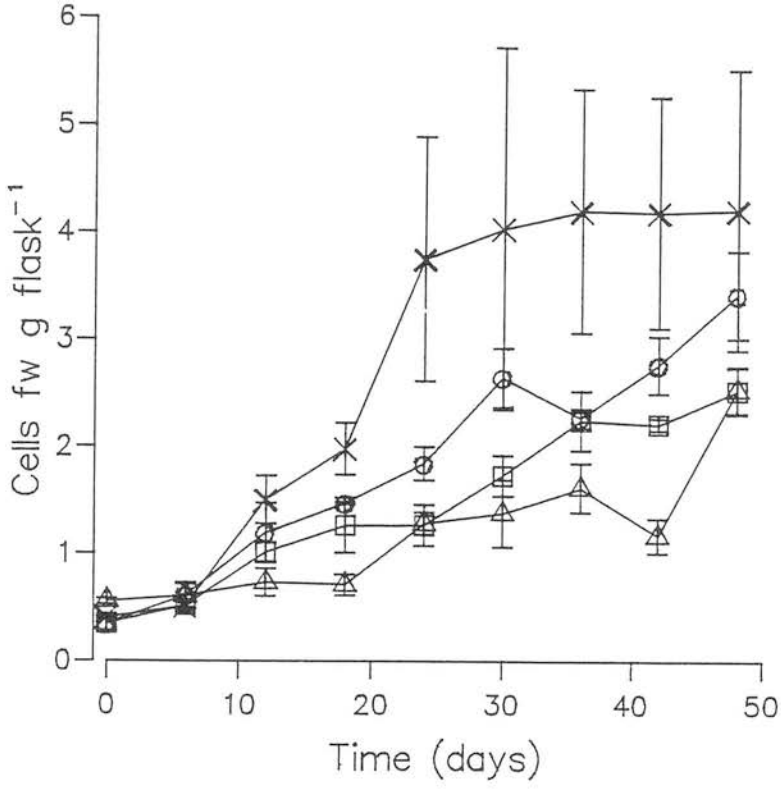
Table 3.5.2 Composition of liquid media with increasing amounts of sucrose as carbon source with $1mgl^{-1}$ 2,4-D and $0.5mgl^{-1}$ BAP at pH 5.8 (* = control)

Medium		
1/2MSB *	1/2MS	**3% sucrose**
1/2MSB6	ditto	**6% sucrose**
1/2MSB9	ditto	**9% sucrose**
1/2MSB12	ditto	**12% sucrose**

Cultures were initiated by inoculating *ca.* 0.5g wet weight of filtered cells into 40ml of medium contained in 250 Erlenmeyer flasks which were then placed on an orbital shaker and incubated under standard culture conditions (2.2.3.4). Three replicates were taken every 6d over an experimental period of 48d. In order to assess growth fw and PCV were determined (see 2.2.4.1 and 2.2.4.3). An estimate of the proportion of pigmented cells was made by pipetting *ca.* a 0.5ml aliquot of suspended cells which was placed on a grid microscope slide (S-7 England finder, Graticulates Ltd., Kent, UK). Counts were performed after the addition of a few drops of a 10% (w/v) sodium carbonate solution which was known to intensify the colour and facilitate the measurements (see 2.2.4.4). Measurements of pH were also made at each sampling interval. In addition the PPPs present in the cells and the medium were extracted and determined quantitatively by HPLC (2.6).

Results presented in Fig. 3.5.14 show the changes in fw of suspended cells cultured in the four different media supplemented with increasing amounts of sucrose (see Table 3.5.2). In the control (1/2MSB, 3% sucrose) there was a lag phase over the first 6d of culture followed by a clear rise until d 24 (10.0 fold increase). Cultures then entered a stationary phase. In 1/2MSB6 the lag phase also lasted for *ca.* 6d and was followed by a regular but slower increase in fw and the stationary phase was reached by d 30, the overall increase was similar to that of the control. Cultures in 1/2MSB9 also showed a lag phase over the first 6d; this was then followed by a regular but slower increase until the end of the experiment with a 7.4 fold, not statistically different from the control (at P=0.05), suggesting nutrients were still available. Finally, cells grown in 1/2MSB12 grew more slowly with a 2.3 fold increase from the onset of culture until d 18, much lower than those obtained with the other three media (1/2MSB, 1/2MSB6 and 1/2MSB9). From d 18 the fw rose further with a final overall increase of 4.6 fold not significantly different from the control (at P=0.05) (see Appendix). Cultures grown in this medium did not exhibit a stationary phase as 1/2MSB9.

Figure 3.5.14 Changes in fw of suspended cells cultured in four different media

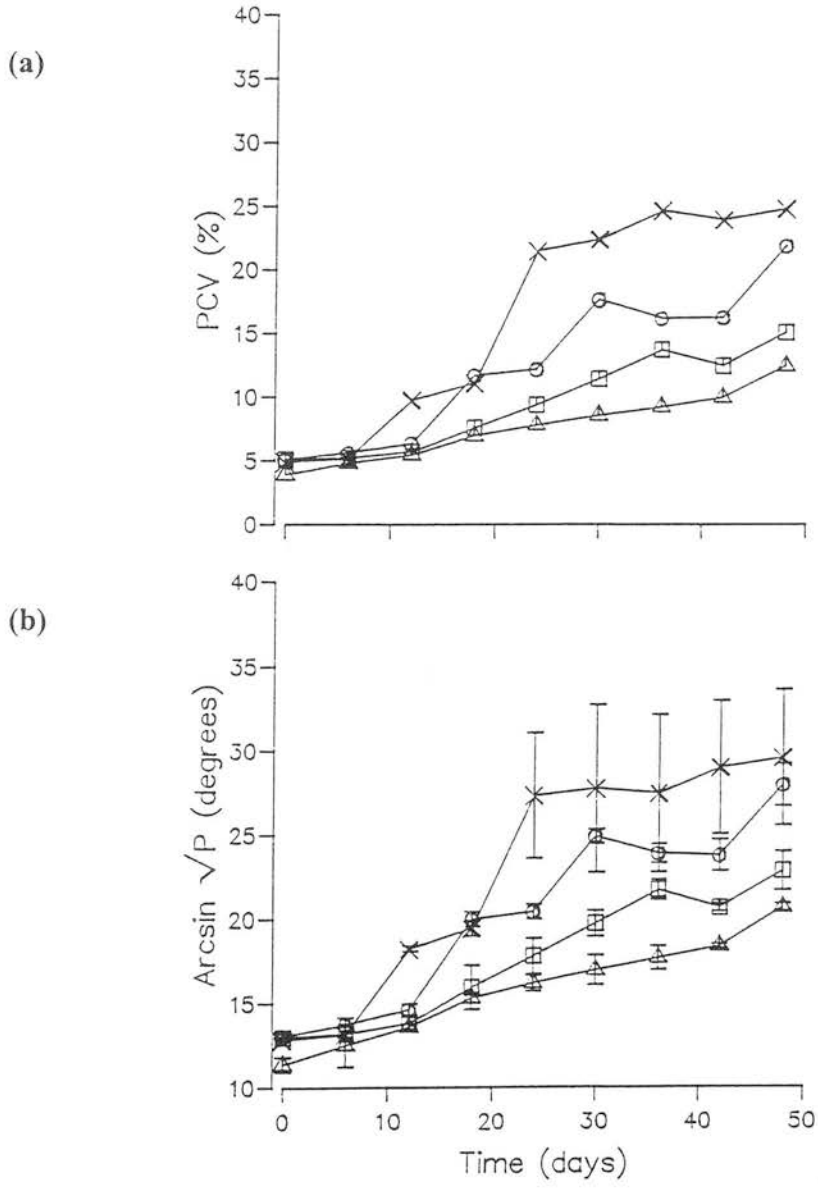


× = 1/2MSB control (3% sucrose), ○ = 1/2MSB6 (6% sucrose), □ = 1/2MSB9 (9% sucrose) and Δ = 1/2MSB12 (12% sucrose)

The maximum fw of 4.2g flask^{-1} was apparently obtained with control cultures (3% sucrose). However, statistical analysis showed no significant differences between the control and treatments possibly because of the large variation between replicates particularly with the control; moreover. significant differences were found between 1/2MSB6 and 1/2MSB9 and 1/2MSB12 on d 24 and d 30. The results for PCV illustrated in Figs. 3.5.15a-b show a similar trend to that observed for fw. It can be seen that there is a lag phase of 6d for all four media. From d 6 the control showed a steady increase until d 24 which was followed by a stationary phase with an overall 5.0 fold increase. In 1/2MSB6 after the initial lag phase the cultures exhibited a steady increase which lasted longer than the control (d 30) which then was followed by a stationary phase with an overall increase of 4.3 fold similar to that reached by the control. Cultures in 1/2MSB9 also showed a lag phase over the first 6d which was followed by a continuous rise until the end of the culture period with a total increase of 2.95 fold. Finally, cells grown in 1/2MSB12 showed a regular but slower increase after the initial lag phase until the completion of the experiment with a total increase of 3.2 fold and no stationary phase, as shown by cells cultured in 1/2MSB9; suggesting, as quoted for the changes in fw that nutrients were still available in these media.

Control cultures were not statistically different to the treatments from d 24 onwards although on d 24 the comparison between control and 1/2MSB12 cultures just missed the difference; however, significant differences are observed between 1/2MSB6 and 1/2MSB9 and 1/2MSB12 from d 24 onwards possibly because of the large variation between replicates particularly with the control (see Appendix). Therefore, it would appear that the higher the level of sucrose the lower the growth; moreover, high levels of sucrose in the media seem to prevent the appearance of a stationary phase suggesting that sucrose is limiting in the control.

Figure 3.5.15 a-b Changes in PCV of suspension cultures grown in four different media **(a)** values expressed as %, each value is the mean of three replicates **(b)** same values after arcsin transformation each value is the mean of three replicates ± se



× = 1/2MSB control (3% sucrose), ○ = 1/2MSB6 (6% sucrose), □ = 1/2MSB9 (9% sucrose) and Δ = 1/2MSB12 (12% sucrose)

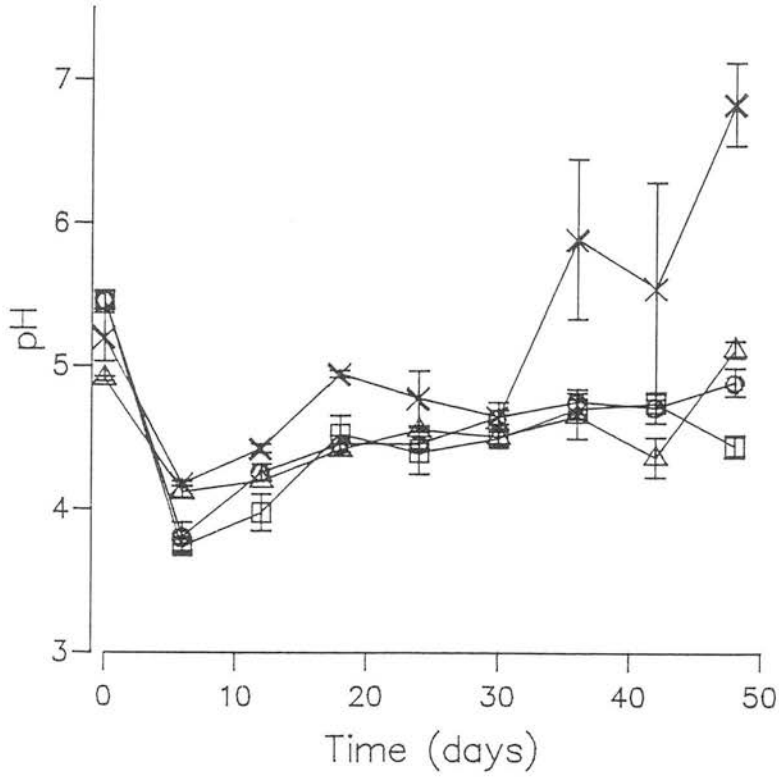
Changes in pH of the four media tested are shown in Fig. 3.5.16 which all display an initial drop during the lag phase. Subsequently the pH drifts up in all treatments reaching *ca.* 5.0 except the control. In contrast the control reaches a pH of 7.0 at d 48 due probably to the release of alkaline substances into the medium following cell autolysis or to changes in the ionic balance.

Results presented in Figs. 3.5.17a-b show the changes in the proportion of pigmented cells in all four cultures from which it can be seen that the proportion of coloured cells for the control remained more or less constant; moreover for the cultures in 1/2MSB6 and 1/2MSB9 there was a slight increase after d 30 although it was not statistically significant, conversely the proportion of pigmented cells in 1/2MSB12 was observed to drift down during the culture period.

Although higher proportions of pigmented cells were observed in 1/2MSB6 and 1/2MSB9, after d 30 statistical analysis showed no difference between these two media and the control; however, at the 12% level there was a lower proportion of pigmented cells and this was statistically significant. It would appear that increasing the amount of sucrose in the medium tend to brings about an elevation in the proportion of coloured cells, although at 12% the effect appears to be inhibitory.

Quantitative analysis of the Et_2O-EtOAc medium extracts of the control failed to detect any PPPs; similar results were obtained from medium extracts of the other three culture media. This contrasts with earlier findings reported in this thesis which showed that most of the [6]gingerol produced in 1/2MSB (control) was released into the medium (see Figs. 3.5.10d-e). The results presented in Figs. 3.5.18a-b show the amounts of [6]gingerol and [6]shogaol present in cell extracts of control, 1/2MSB6, 1/2MSB9 and 1/2MSB12 cultures and presents a complex picture. In the control the amounts of these metabolites increased from the initiation of culture reaching maximum values at d 18.

Figure 3.5.16 Changes in the medium pH of cells cultured in four different media. Each value is the mean of three replicates ± se



× = 1/2MSB control (3% sucrose), ○ = 1/2MSB6 (6% sucrose), □ = 1/2MSB9 (9% sucrose) and Δ = 1/2MSB12 (12% sucrose)

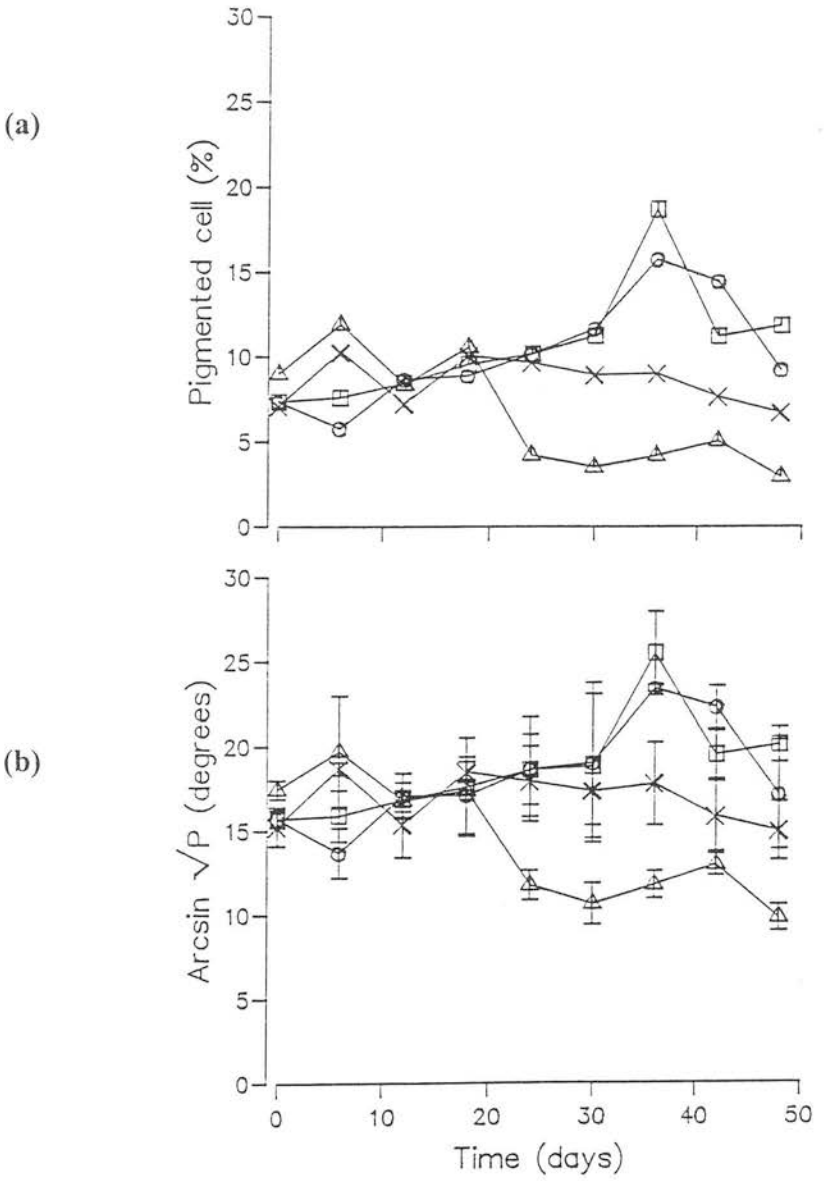
Subsequently the amounts tended to decrease reaching the lowest value at the end of the culture period. This pattern did not parallel the changes in the proportion of pigmented cells (see Figs. 3.5.17a-b). It can also be seen that the amount of [6]shogaol was much higher than [6]gingerol. From these results it would appear that the synthesis of these metabolites takes place at an early stage of growth (18d) when the cultures were still showing active growth (see Fig. 3.5.14) in contrast to the initial findings which showed the accumulation of [6]gingerol at d 35-40 when growth had ceased (see Figs. 3.5.10a and 3.5.10e). On 1/2MSB6 the amount of [6]shogaol was also higher than [6]gingerol. From Figs. 3.5.18a-b it can be seen that [6]shogaol rose from the onset of culture until d 6 (lag phase, see Fig. 3.5.14). After d 18 the amounts of both compounds fell to reach the lowest values recorded at the end of the experiment. A comparison between this latter result and the proportion of pigmented cells does not present a positive correlation since the highest proportion of pigmented cells was achieved at the later stages of growth d 36-42 (see Figs. 3.5.17a-b). Similarly, in 1/2MSB9 cultures the amount of [6]shogaol was also much higher than [6]gingerol. Here an initial increase at d 6 in the amount of [6]shogaol was recorded which then remained fairly constant until d 30. However, [6]gingerol increase from d 18 which also remained almost constant until d 30 followed by a final drop at the end of the experiment for both compounds. As with 1/2MSB6 the highest proportion of pigmented cells which was observed in the later stages of growth did not correspond with high amounts of PPPs (see Figs. 3.5.17a-b). Finally, the yield of these metabolites on 1/2MSB12 was close to zero throughout the culture period which would indicate an inhibitory effect on the production of PPPs at this high level of sucrose. Also it should be noted that as for 1/2MSB6 and 1/2MSB9, the high proportion of pigmented cells did not coincide with a high yield of these metabolites.

Therefore, it would appear that the control provides better growth with maximum fw values, also the accumulation of PPPs was higher in the control. Conversely, it would appear that the high levels of sucrose tested alter the kinetics of synthesis of

PPPs. Also a decrease in cell biomass was observed at the highest sucrose levels; moreover, especially the 12% level which gave the lowest growth was also inhibitory for the production of these metabolites. There is also no positive correlation between the proportion of pigmented cells and the accumulation of PPPs in cultured cells. In addition, 6% and 9% sucrose level seem to increase the proportion of pigmented cells compared to the control particularly on d 36-42 although the values were very close to the significant level (at P=0.05).

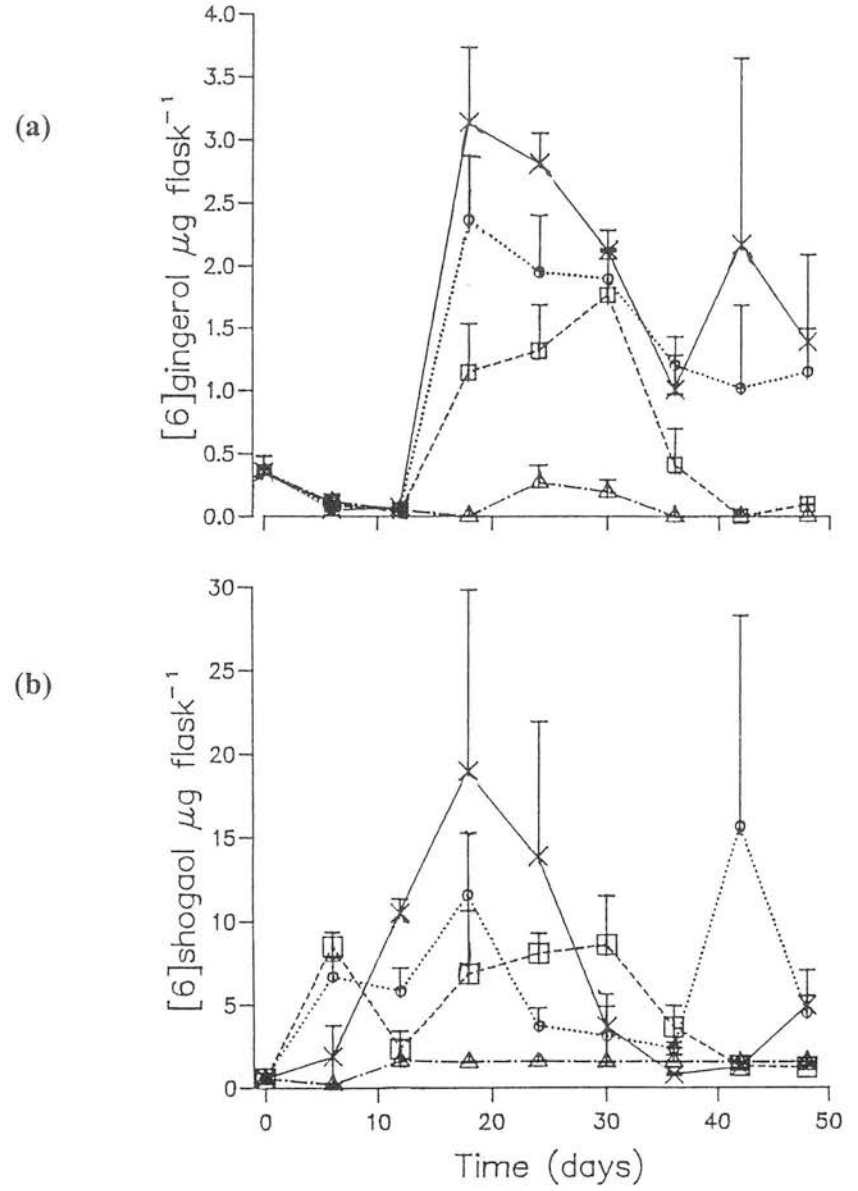
Consequently, in view of these results it was decided to employ 1/2MSB (control) as a standard medium for further experiments. Bearing in mind the changes in PPPs accumulation and the appearance and disappearance throughout the growth culture, it was decided to investigate the fate of added [6]gingerol to suspension cultures to find possible explanation of these uncommon observations.

Figure 3.5.17 a-b Changes in the proportion of pigmented cells in four different cultures **(a)** values expressed as %, each value is the mean of three replicates **(b)** same values after arcsin transformation each value is the mean of three replicates ± se



× = 1/2MSB control (3% sucrose), ○ = 1/2MSB6 (6% sucrose), □ = 1/2MSB9 (9% sucrose) and Δ = 1/2MSB12 (12% sucrose)

Figure 3.5.18 a-b Amount of **(a)** [6]gingerol and **(b)** [6]shogaol in suspended cells cultured in four different media. Each value is the mean of three replicates ± se



× = 1/2MSB control (3% sucrose), ○ = 1/2MSB6 (6% sucrose), □ = 1/2MSB9 (9% sucrose) and Δ = 1/2MSB12 (12% sucrose)

3.6 Studies on the fate of [6]gingerol administered to suspension cultures of *Z.officinale*

It has been shown in a number of experiments in this thesis that [6]gingerol and related compounds appear and disappear during a culture cycle and that some partitioning exists between cells and medium. It is therefore likely that the amount of these compounds present at any one time is the result of balance between synthesis and degradation as well as transport away from the site of synthesis. In order to discover whether suspended cells are capable of degrading, modifying, and transporting [6]gingerol it was decided to follow the fate of this compound when added to cell cultures during the culture cycle. Cultured cells of different ages and developmental stages were chosen; 15d old cells are at the midpoint of growth, when the production of [6]gingerol was low, and 35 d old cells which had reached the stationary phase when accumulation of [6]gingerol was marked (see Figs. 3.5.10a-b and 3.5.10e).

125ml Erlenmeyer flasks were prepared each containing 20ml of 1/2MSB and either 15d or 35d old cells with 1.3 and 1.8g of wet weight respectively. To each flask was added 7.6µl of a stock solution of [6]gingerol in MeOH [3.87mg in 1ml] corresponding to 29.45µg. The flasks were then sealed with a double layer of sterilised aluminium foil, shaken by hand to ensure even distribution of the added compound and then placed on an orbital shaker under standard cultures conditions (2.2.3.4).

Three replicates were sampled immediately after the administration of the [6]gingerol and then at 3, 12, 24 and 48h. Medium and cells were separated by filtration through a Whatman No. 1 filter paper; subsequently, cells and medium were extracted as described previously (see 2.3.2.2, 2.3.2.2.1), also at the initiation of this experiment three replicates of control cultures (20ml volume of cultures grown in

1/2MSB) were sampled. Quantitative analysis was performed using HPLC as described in section (2.6).

The results presented in Table 3.6.1 show the amounts of [6]gingerol and [6]shogaol extracted from 15d old cultures to which 29.45μg of [6]gingerol had been added initially. It can be seen from the results depicted in Table 3.6.1 that the cells at time 0h contain amounts of [6]gingerol similar to the control; but the amount of [6]shogaol present was almost double to that shown by the control but still very small compared with the initial amount of [6]gingerol added to the cultures. Therefore, it may be expected that the rest of the [6]gingerol would be present in the medium. However, the total amount of these two compounds in the cultures (cells + medium) is still only a small part of the 29.45μg added to each flask. Over the experimental period the total amount of both [6]gingerol and [6]shogaol fluctuate and at 48h reaches approximately 50% of that added at time 0h. It would appear that this compound has become bound to membranes, cell walls or components in the medium preventing its extraction. It is very unlikely that at 0h time much of the [6]gingerol would have been metabolised although this would presumably occur as the experiment progressed. The results presented in Table 3.6.2 for 35d old cultures (stationary phase) show much higher amounts of [6]gingerol and [6]shogaol in the cells at time 0h than the control. Indeed the total amount of [6]gingerol and [6]shogaol is equivalent to the amount added initially. Subsequently the total amount of [6]gingerol and [6]shogaol recovered at 3, 12, 24 and 48h was much less than at time 0h but similar to the control.

Bearing in mind that 29.45μg of [6]gingerol was added to each flask and very low amounts of this metabolite were recovered in all but 0h in 35d old cultures from both cells and medium the whereabouts of the added compound is a mystery. Attempts were now made to check the extraction efficiency and to explore the possibility of glycosylation or binding to membranes or cell walls or components of the medium.

Table 3.6.1 Amounts of [6]gingerol and [6]shogaol present in cells and medium of 15d old suspended cells cultured in 20ml 1/2MSB after addition of 29.45µg of [6]gingerol. Each value is the mean of three replicates ± se (µg flask^{-1})

	0h	3h	12h	24h	48h	control
[6]gingerol cells	1.63±0.01	2.33±0.13	2.33±0.64	1.85±0.60	7.80±2.35	1.85±0.40
[6]gingerol medium	1.51±0.89	nd	nd	nd	nd	nd
Total per culture	3.14	2.33	2.33	1.85	7.80	1.85
[6]shogaol cells	0.70±0.16	5.61±0.86	0.51±0.25	3.77±1.64	6.51±1.10	0.37±0.37
[6]shogaol medium.	0.39±0.39	nd	nd	nd	nd	nd
Total per culture	1.09	5.61	0.51	3.77	6.51	0.37
Total [6]gingerol and [6]shogaol per culture	3.23	7.94	2.84	5.62	14.31	2.22

nd= not detected

Table 3.6.2 Amounts of [6]gingerol and [6]shogaol present in cells and medium of 35d old suspended cells cultured in 20ml 1/2MSB after addition of 29.45μg of [6]gingerol. Each value is the mean of three replicates ± se (μg flask^{-1})

	0h	3h	12h	24h	48h	control
[6]gingerol cells	12.26±6.3	1.65±0.51	2.13±0.40	2.42±0.14	1.58±0.79	1.77±0.25
[6]gingerol medium.	6.52±0.47	nd	nd	nd	nd	1.16±0.58
Total per culture	18.78	1.65	2.13	2.42	1.58	2.93
[6]shogaol cells	11.30±5.53	2.37±0.46	3.44±0.25	2.50±0.93	3.35±3.08.	1.47±1.05
[6]shogaol medium.	0.20±0.08	0.14±0.07	0.15±0.02	0.15±0.02	0.19±0.02	0.34±0.11
Total per culture	11.50	2.51	3.58	2.65	3.54	1.81
Total [6]gingerol and [6]shogaol per culture	30.28	2.65	5.71	5.15	5.12	4.74

nd= not detected

3.6.1 Determination of extraction efficiency

In order to investigate the efficiency with which the PPPs were extracted from the medium using a solvent mixture of (1:1) (v/v) Et_2O-EtOAc as described in 2.3.2.2.1 two different amounts of [6]gingerol (29.45 and 60μg) taken from a MeOH solution (3.87mg ml^{-1}) were added to 20ml of both, fresh autoclaved 1/2MSB medium and 25d old conditioned medium employing three replicates.

After the addition of [6]gingerol, the flasks were shaken by hand to ensure the even distribution of the compound in the medium and *ca.* after 10-15min the extraction of this metabolite was carried out 3x with equal volumes of a mixture 1:1 (v/v) Et_2O-EtOAc as described in 2.3.2.2.1. The combined organic fractions were evaporated to dryness under vacuum over a warm water bath at 35°C; and the residue dissolved in 1ml HPLC MeOH, filtered through a 0.45μm Nylon membrane and analysed by HPLC (see 2.6)

The results presented in Table 3.6.3 show an extraction efficiency of 74.10% when 29.45μg of [6]gingerol was added to 20ml of fresh autoclaved 1/2MSB medium. Similarly, an efficiency of 73.10% was achieved when 60μg of [6]gingerol was added to the same medium. Therefore, it would appear that the efficiency of extraction from freshly autoclaved medium is not affected by the concentration of [6]gingerol added. These results indicate that the method of extraction practised is satisfactory although not a hundred percent efficient. Thus if a lower recovery is obtained after extraction from conditioned medium it would imply that [6]gingerol reacts with compounds present in the medium arising from the cultured cells and therefore decreases the efficiency of extraction.

Indeed the results presented in Table 3.6.3 show that when 29.45μg and 60μg of [6]gingerol were added to 25d conditioned medium the extraction efficiency achieved (*ca.* 56%) was much lower than that obtained with freshly autoclaved medium (*ca.* 74%). Moreover, the amount of [6]gingerol added does not seem to affect its recovery

since an almost identical percentage of efficiency was achieved in both instances. Therefore, in view of these latter results, it would seem that certain compounds present in the medium, released or metabolised by the cells, have reacted with [6]gingerol complexing or binding with the molecule perhaps by glycosylation and this impairs extraction of the added compound. This is consistent with the interpretation of the results, suggesting they may be bound to cell walls or membranes. Therefore, two possibilities should be considered firstly, the formation of bound forms/conjugates formed with compounds present in the medium and secondly, binding to either cell walls or membranes or both. These possibilities are now investigated.

Table 3.6.3 Efficiency of extraction of **(a)** 29.45µg and **(b)** 60µg of added [6]gingerol to 20ml of freshly autoclaved and 25d old conditioned media. Each value is the mean of three replicates ± se

	Amount recovered (µg flask^{-1})	**Efficiency (%)**
Freshly autoclaved medium	21.81 ± 1.75 (a)	74.10 (a)
	43.84 ± 3.47 (b)	73.10 (b)
25 d conditioned medium	16.50 ± 0.65 (a)	56.03 (a)
	33.79 ± 2.70 (b)	56.31 (b)

3.6.2 Identification of bound forms of phenolic pungent compounds in cultures of *Z.officinale*

The aim of these experiments was to investigate the possible presence of bound forms of the PPPs in ginger extracts since the recovery of [6]gingerol added to cultures was poor.

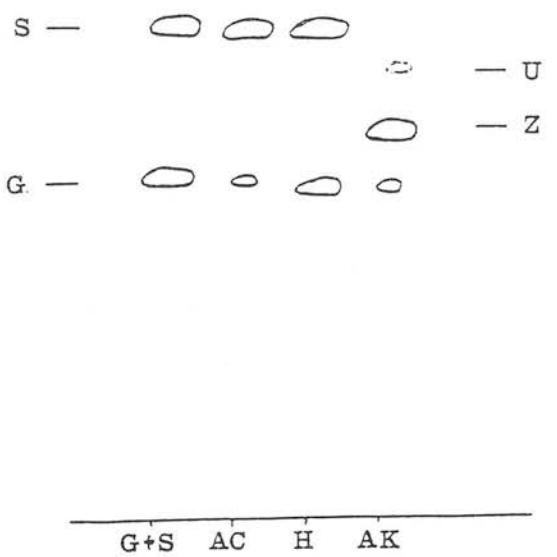
Reports in the literature have shown that conjugation of phenolics in plants removes the toxicity of these compounds (Harborne, 1973; Brown, 1981) and enables storage. For example Sukrasno and Yeoman (1993) have reported the presence of glycosylated phenolics in fruits of chilli pepper. However, there are no reports of glycosylates of the ginger phenolics, although Wu *et al.* (1990) have shown that some of the glycosidically bound aroma compounds are present in essential oils. It is also known that [6]gingerol is converted to zingerone after alkaline hydrolysis via a retroaldol condensation (Connell and McLachlan, 1972 and McHale *et al.* 1989) and that the conversion of [6]gingerol to [6]shogaol takes place during acid hydrolysis via dehydration (Connell and McLachlan, 1972 and McHale *et al.* 1989).

The general strategy followed in this series of experiments was to subject extracts from the ginger cultures to several forms of hydrolysis including the use of alkali, acid and enzymes and then to examine the products.

First of all a preliminary experiment was carried out to determine whether [6]gingerol was affected by alkaline or acid hydrolysis or by heat treatment. The alkaline and acid hydrolysis of 193.75μg of [6]gingerol dissolved in 100μl of MeOH were performed as described in Section 2.8. The products of hydrolysis were then analysed by TLC using plates with a fluorescent indicator after development with solvent system II (see 2.5). The spots were visualised under UV light as described in 2.5.2.1. It can be seen from the results presented in Fig. 3.6.1 that after alkaline hydrolysis most of the [6]gingerol was converted to zingerone with an R_f value of 0.44. Although an authentic standard for this compound was not available it has been

assumed tentatively using reports in the literature, that this compound is zingerone. Similarly, after acid hydrolysis most of the [6]gingerol was converted to [6]shogaol which co-chromatographs with pure [6]shogaol on TLC. In addition to the hydrolytic treatments a sample of [6]gingerol was subjected to 120°C for 1h to assess its thermal stability. It can be seen from Fig. 3.6.1 that following heat treatment some of the [6]gingerol has been converted to [6]shogaol.

Figure 3.6.1 TLC developed with solvent system II showing the products of alkaline and acid hydrolysis and the heat treatment of [6]gingerol. Spots were visualised under UV light. (G=[6]gingerol, S=[6]shogaol, AC=acid hydrolysis, H=heat treatment, AK=alkaline hydrolysis, Z=zingerone ?, U= unknown)



The results obtained from this preliminary experiment show that the hydrolytic conditions employed would, in addition to the rupturing of the ester and glycosidic bonds, also produce a range of compounds derived from [6]gingerol.

3.6.2.1 Alkaline hydrolysis of the aqueous fraction from liquid medium

Phenolic compounds bound via an ester linkage will be released by alkaline hydrolysis by cleavage of these bonds.

After LLE the volume of the aqueous fraction from the medium of 15d cultures to which 29.45μg of [6]gingerol had been administered was reduced to *ca.* 5-10ml under vacuum and then subjected to alkaline hydrolysis with N KOH overnight at room temperature giving a pH of 10.5. The products of hydrolysis were then acidified by the addition of a few drops of N HCl, liquid-liquid extracted with Et_2O-EtOAc as described in 2.3.2.2.1 and then analysed by TLC and/or by HPLC. TLC plates containing a fluorescent indicator were employed and these were developed using solvent system II. Spots were visualised under UV light and then sprayed with Folin-Ciocalteu reagent to assess the phenolic nature of the spots (see 2.5.2). Authentic standards of [6]gingerol and [6]shogaol together with a sample of the product of alkaline hydrolysis of [6]gingerol *i.e.* zingerone (see 2.6.2) were loaded onto the TLC plate to enable identification of the hydrolysis products.

It can be seen from the results presented in Fig. 3.6.2 that alkaline hydrolysis of the aqueous fraction produces a number of spots some of which co-emigrated with zingerone and [6]shogaol with R_f values of 0.44 and 0.53 respectively (see 3.6.2). After spraying with Folin-Ciocalteu some of the spots previously recorded under UV light reacted with this reagent producing very faint spots indicating their phenolic nature. These results are consistent with the explanation that some of the phenolic were bound to other moieties via ester bonds and this would perhaps explain the poor recovery obtained after LLE of the medium (see 3.6).

3.6.2.2 Emulsin hydrolysis of the aqueous fraction from liquid medium

Phenolic compounds bound via ester link would be also cleaved after emulsin hydrolysis providing a similar reaction product as the alkaline hydrolysis described above.

After LLE the volume of the aqueous fraction from the medium of 15d cultures to which 29.45μg of [6]gingerol had been administered was evaporated to dryness under vacuum and then redissolved in 5ml of 0.05M acetate buffer (pH 5.0). To this 1.5mg of emulsin (β-glucosidase from almond, Sigma) was added and the mixture incubated over a warm water bath at 35°C for 24h. The products of hydrolysis were then LLE with Et_2O-EtOAc (2.3.2.2.1) and then analysed by TLC and/or HPLC. TLC was developed as described earlier (3.6.2.1) and HPLC as described in 2.6. Analysis of the emulsin hydrolysis products both by TLC and HPLC failed to detect any of the PPPs in contrast with the results obtained after alkaline hydrolysis which indicated the presence of compounds conjugated via ester bonds perhaps indicating a lack of activity of the enzyme employed.

3.6.2.3 Acid hydrolysis of the aqueous fraction from liquid medium

It has now been shown that gingerol and/or related compounds appear to be conjugated via ester bounds to compounds present in the medium since after alkaline hydrolysis some of these compounds were detected in a free state (see 3.6.2.1). It was now decided to investigate the possibility of these phenols being conjugated via O-glycosidic bonds.

Phenolics conjugated to other moieties via O-glycosidic bonds are released after acid hydrolysis which also cleaves ester bounds. Samples of the aqueous fraction of 15d cultures which had been administered with 29.45μg of [6]gingerol were subjected to acid hydrolysis to discover the presence of phenolics bound to sugars.

After LLE the volume of the aqueous fraction from the liquid medium of 15d cultures administered with 29.45µg of [6]gingerol was reduced to *ca.* 5-10ml under vacuum and then subjected to acid hydrolysis in 2 N HCl at 80-90°C for 2h. After the samples had cooled down LLE was carried out with Et_2O-EtOAc as described previously and samples analysed by TLC and/or by HPLC (see 2.6.2.1). Authentic standards of [6]gingerol and [6]shogaol together with a sample of the product of alkaline hydrolysis of [6]gingerol *i.e.* zingerone (see 3.6.2) were loaded onto the TLC plate to enable identification of the hydrolysis products.

The results presented in Fig. 3.6.3 show that after acid hydrolysis [6]shogaol with an R_f value of 0.53 was visualised both under UV and with Folin-Ciocalteu reagent at an amount suggesting conversion from the added [6]gingerol. In one of the replicates [6]gingerol with a R_f value of 0.41 was also visualised under UV light but not after sprayed with Folin-Ciocalteu reagent, likely due to the low amount present, showing that all of this phenolic compound had not been converted to [6]shogaol by hydrolysis.

It would appear that the results presented in 3.6.2.1 and 3.6.2.3 provide evidence that some of the PPPs in cultures are conjugated both via ester and O-glycosidic bonds to other compounds present in the medium. This would account for the low recovery of [6]gingerol after LLE of the medium (see 3.6.).

It was now decided to discover whether these bound compounds were present in the remaining aqueous fraction of 15d cultures administered with 29.45µg of [6]gingerol (12, 24, and 48h after addition) and to determine how much [6]gingerol and related compounds was extracted to assess the recovery rate after acid hydrolysis.

Acid hydrolysis was conducted as described before and the samples were LLE with Et_2O-EtOAc and analysed by HPLC (see 2.6). The results presented in Table 3.6.4 show the amount of [6]gingerol and [6]shogaol recovered after hydrolysis. It can be seen that for the 12h sample no gingerol was recovered although 2.35µg of

[6]shogaol was present which represents approximately 8% of the total amount of [6]gingerol added initially to the cultures (29.45μg). After 24 and 48h the hydrolysed samples contained 0.55μg and 0.13μg of [6]gingerol respectively; also the amounts of [6]shogaol recovered were much higher than at 12h 5.28μg and 4.05μg at 24 and 48h respectively which accounts for *ca.* 20% and 14% of added [6]gingerol (29.45μg).

These results show that approximately 14% of all the PPPs have been recovered following acid hydrolysis and this could explain the poor recovery of these compounds prior hydrolysis. It is probable that similar recovery rates would be obtained from 35d cultures.

Figure 3.6.2 Phenolic pungent principles detected after alkaline hydrolysis of the aqueous fractions of 15d suspension cultures administered with 29.45µg of [6]gingerol separated by one dimensional TLC using solvent system II; the spots were visualised both under short UV light (dotted spots) and with Folin-Ciocalteu (continuous spots). abc=replicates, st=standard mixture, G=[6]gingerol, S=[6]shogaol, Z=zingerone ?, u= unknown

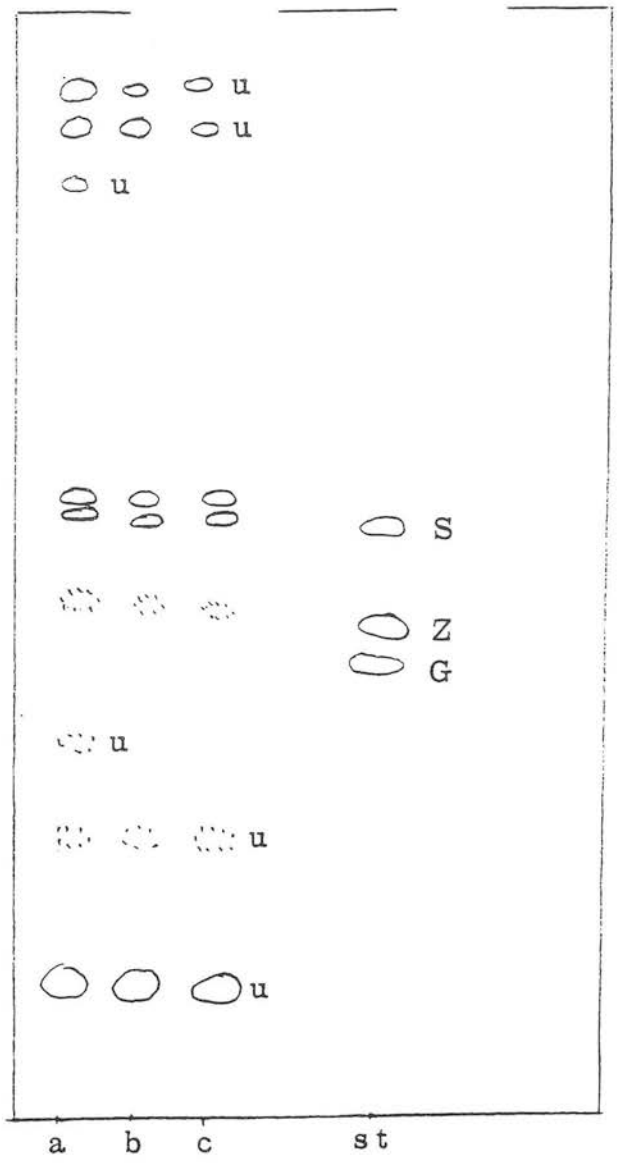


Figure 3.6.3 Phenolic pungent principles detected after acid hydrolysis of the aqueous fraction of 15d suspension cultures administered with 29.45µg of [6]gingerol separated by one dimensional TLC using solvent system II; the spots were visualised both under UV light (dotted spots) and with Folin-Ciocalteu (continuous spots). abc=replicates, st=standard mixture, G=[6]gingerol, S=[6]shogaol, Z=zingerone ?, u=unkown

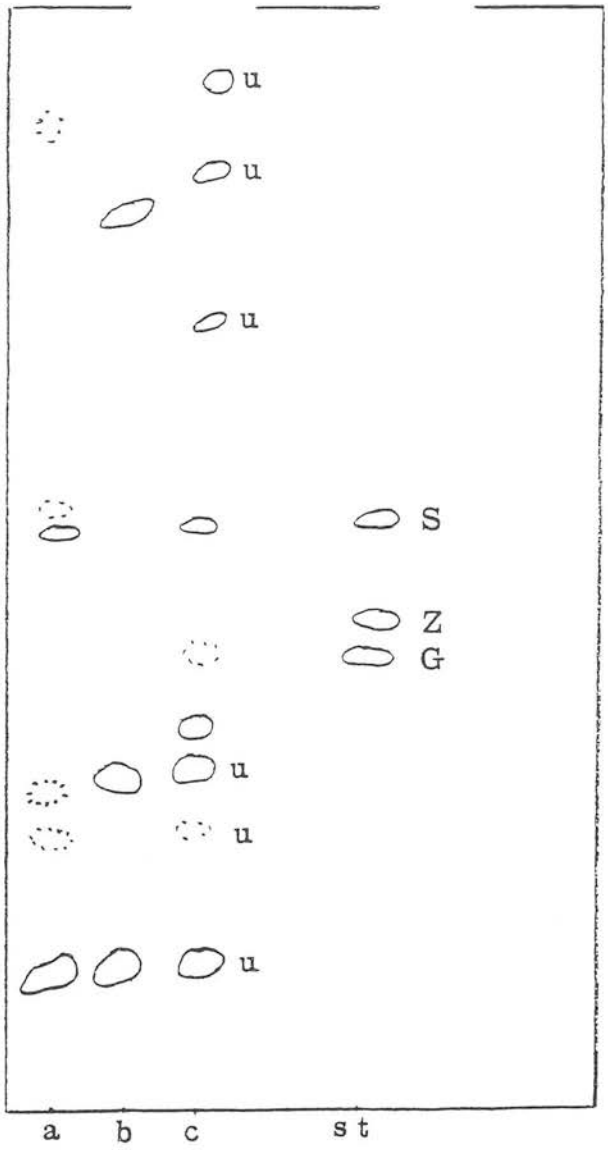


Table 3.6.4 Recovery of [6]gingerol and [6]shogaol following acid hydrolysis of the aqueous fractions of 15d cultures administered with 29.45μg of [6]gingerol. Each value is the mean of three replicates ± se (μg flask^{-1})

	12h	24h	48h
[6]gingerol	nd	0.55±0.55	0.13±0.13
[6]shogaol	2.35±0.79	5.28±1.65	4.04±1.89
Total	2.35	5.83	4.17
% recovery from the initially added [6]gingerol	8.0%	19.80%	14.15%

nd = not detected

3.6.2.4 Acid hydrolysis of the cell residue

It is possible that bound forms of the added [6]gingerol may be present in the cell residue. In order to investigate this possibility two kinds of acid hydrolysis (mild and vigorous) were performed.

125ml Erlenmeyer flasks were prepared each containing 20ml of 1/2MSB with *ca.* 1.8g of fw of 31d old cells. To each flask was added 8μl of a stock solution of [6]gingerol in MeOH [3.87mg in 1ml] corresponding to 31μg. The flasks were then sealed with a double layer of sterilised aluminium foil, shaken by hand to ensure even distribution of the added compound and then placed on an orbital shaker under standard cultures conditions (2.2.3.4).

Three replicates were sampled 3h after the administration of [6]gingerol. Medium and cells were separated by filtration through a Whatman No. 1 filter paper; subsequently, cells and medium were extracted as described previously (see 2.3.2.2, 2.3.2.2.1), and after acetone extraction of the cells the cell residue was retained for

acid hydrolysis. Three replicates of the control cultures (without the addition of [6]gingerol or MeOH) were treated in exactly the same way.

The cell residue *ca.* 0.15g dw was first subjected to mild acid hydrolysis in M NH_4Cl in a boiling water bath for 1h. The reaction mixture was then filtered and the aqueous fraction (which would contain any released compound after hydrolysis) liquid-liquid extracted 3x with Et_2O-EtOAc as described previously. The samples were prepared and analysed by TLC using solvent system II and the spots visualised both under UV light and with Folin-Ciocalteu reagent and also by HPLC (see 2.5 and 2.6).

The cell debris (which had been acid hydrolysed in M NH_4Cl) remaining after filtration was then subjected to vigorous acid hydrolysis in 2N HCl in a boiling water bath for 1h and the samples prepared as described above.

Analysis of the samples from both acid hydrolysates by TLC and HPLC failed to show the presence of [6]gingerol or [6]shogaol. However, samples from the vigorous acid hydrolysis analysed by TLC using solvent system II showed a very dark spot (unknown compound) with an R_f value of 0.50 after spraying with Folin-Ciocalteu reagent. This was different from [6]gingerol (0.38) and [6]shogaol (0.66) and was present in both control and treatment which suggests that this compound did not originate from the added [6]gingerol.

From these results presented in 3.6.2 it would seem that the [6]gingerol added to cell cultures conjugates via ester and O-glycosidic bonds with compounds present in the medium; however, no bound forms were found in the cell residue. These results could explain the poor recovery of added [6]gingerol.

In another attempt to improve product yield sunflower oil was added to suspension cultures since report in the literature show that product yield of lipid soluble compounds could be enhanced by providing a lipid sink; accordingly in the next

section the effects of the addition of sunflower oil upon the accumulation of PPPs in suspension cultures are outlined.

3.6.3 Effects of the addition of sunflower oil upon the accumulation of phenolic pungent principles in suspension cultures

In section 3.2 it was established that the yellow pigmented cells were the repositories of [6]gingerol, lipid probably other PPPs and a range of flavonoid-like compounds.

These coloured cells were also observed in suspension cultures although there was no direct correlation between the number of pigmented cells and the amount of [6]gingerol present (see 3.5.4).

As these compounds appear to be present in storage lipid it might be possible to increase the production of [6]gingerol in cell cultures by providing a 'lipid sink' external to the cells.

There are some reports in the literature of yield increases of lipid soluble compounds in cell cultures by providing oil to the growth medium. For example Bisson *et al.* (1983) report that the essential oil production in *Matricaria chamomilla* cultures was enhanced when a triglyceride phase was added to the cultures; Berlin *et al.* (1984) report that the addition of miglyol to cell cultures of *Thuja occidentalis* was useful for the release of monoterpenes and for the accumulation of very volatile monoterpenes which cannot accumulate in water.

Before studying whether [6]gingerol production could be stimulated by the addition of sunflower oil a preliminary experiment was conducted to assess the recovery of [6]gingerol from the oil. Sunflower oil purchased in a local supermarket was sterilised in an autoclave at 121°C at a steam pressure of 15 psi for 20min. A mixture of Et_2O-EtOAc which was used routinely for LLE of the PPPs was not

suitable for the extraction of [6]gingerol and related compounds from sunflower oil. However, MeOH an organic solvent of higher polarity than Et_2O-EtOAc was suitable producing two layers when mixed with oil.

In order to study the suitability of this solvent in the extraction of [6]gingerol from sunflower oil, 60μg of [6]gingerol from a 3.875mg ml^{-1} MeOH stock solution was administered to 5ml of sterilised sunflower oil. The mixture was then hand shaken to ensure even distribution of the added compound and then extracted 3x with double the volume of MeOH. To assist extraction the mixture was shaken vigorously so producing a cloudy emulsion. Separation was achieved by centrifugation at 3000rpm (1.4 xg) for 5 min. After centrifugation the MeOH fraction (top layer) was taken, evaporated to dryness under vacuum over a warm water bath at 35°C and the residue taken up in 1ml MeOH (HPLC grade), filtered through a nylon filter and analysed by HPLC (see 2.6).

The results presented in Table 3.6.5 show that an average recovery of *ca.* 72.8% was achieved, confirming the suitability of MeOH for the extraction of [6]gingerol

Table 3.6.5 Recovery of [6]gingerol (60μg) by MeOH from 5ml of sunflower

Replicate	Amount recovered (μg)	Recovery (%)
1	44.47	74.11
2	42.98	71.63

Following this successful result, an experiment was conducted with 40ml of a 22d old suspension culture grown in 1/2MSB medium. 5ml of sterilised sunflower oil was added aseptically to each of the 12 flasks. Three replicates were sampled at 5d intervals until 42d. In parallel, 3 replicates of controls (cultures of the same age but

lacking oil) were also sampled at the same intervals to assess the effects of added oil on culture growth and accumulation of the PPPs.

At each sample time the pH of the medium was determined and the different fractions (cells, medium and oil) were separated by centrifugation at 3000rpm (1.4xg) for 5-8min. After centrifugation the oil fraction, which was located at the top of the tube, was transferred to centrifuge tubes and extracted 3X with MeOH as described earlier. The samples were then prepared for HPLC and analysed (see 2.6).

The remaining two fractions (cells and medium) were separated by filtration (see 2.3.2.2). After recording the fw, the cells were ground up and extracted with acetone at room temperature for 3h and the extracts prepared for HPLC (see 2.3.2.2). The medium was extracted 3X with Et_2O-EtOAc as described earlier. Cells and medium of the control replicates were treated in the same way.

The results presented in Fig. 3.6.4a show the changes in fw of cultures with and without oil. It can be seen that the addition of oil did not affect growth. Similarly, the changes in pH presented in Fig. 3.6.4b are also similar.

Quantitative analysis of the medium extract failed to detect any PPPs similar to the results described in 3.5.4. Conversely, the results presented in Table 3.6.6 show the presence of [6]gingerol and [6]shogaol in the cells of both control and in the oil.

It can be seen from Table 3.6.6 that some of the gingerol and shogaol is present within the oil, in one instance about 45% of the total. However, the addition of oil did not appear to boost the overall production of these metabolites.

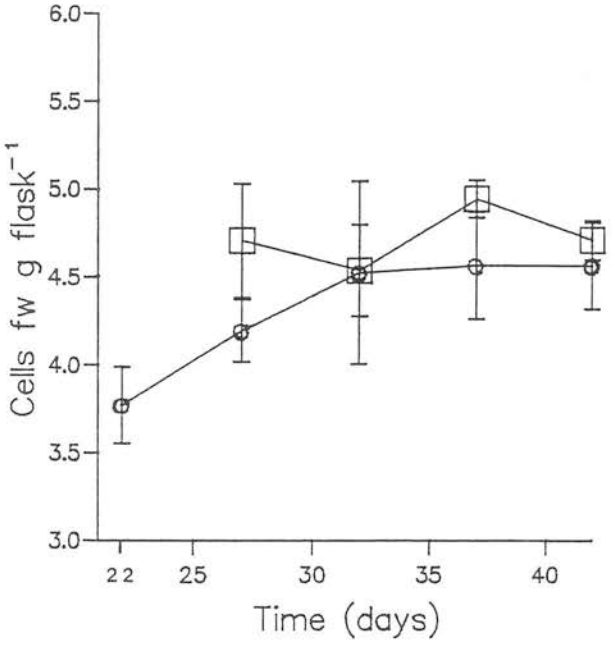
Therefore, it would appear that some of the [6]gingerol and [6]shogaol is within the cells presumably in lipid; however the addition of oil does not seem to stimulate the synthesis and accumulation of these compounds.

The different strategies undertaken to improve yield and culture condition have led to 1/2MSB as the best culture medium so in the next sections (3.7 and 3.8) attempts

were made to investigate the biosynthetic pathway leading to [6]gingerol employing radioactive putative intermediates administered to rhizome pieces and suspension cultures of *Z.officinale*.

Figure 3.6.4 a-b Changes in **(a)** fw and **(b)** medium pH of control (○) and treatment (cultures with 5ml of sterilised sunflower oil)(□). Each value is the mean of three replicates ± se

(a)



(b)

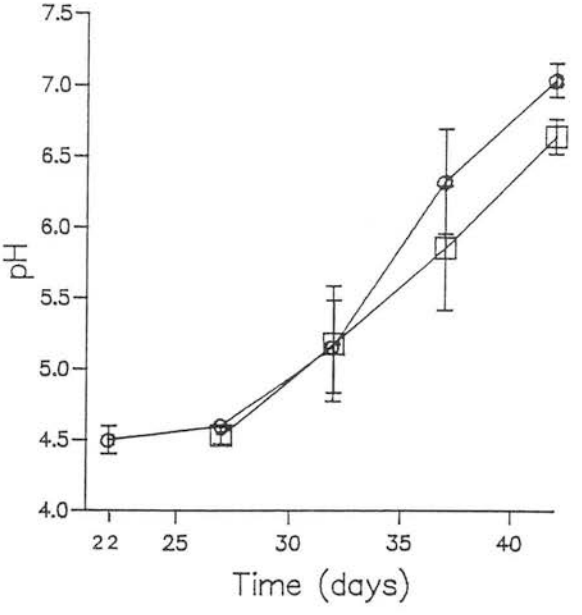


Table 3.6.6 Amounts of [6]gingerol (G) and [6]shogaol (S) present in the cells of control and treatment and in the oil fraction. Each value is the mean of three replicates ± se (*μg flask^{-1}, ** μg oil^{-1})

	d 27		d32		d37		d42	
	[6]gingerol	[6]shogaol	[6]gingerol	[6]shogaol	[6]gingerol	[6]shogaol	[6]gingerol	[6]shogaol
Control*	0.193±0.06	3.05±0.60	0.26±0.26	3.28±0.75	0.08±0.03	3.33±0.17	0.04±0.02	2.28±0.79
Total G+S *		3.24		3.54		3.42		2.32
Treatment*	0.05±0.02	1.94±0.09	0.06±0.04	1.94±0.23	0.12±0.06	2.32±0.20	0.28±0.11	0.4±0.23
Total G+S *		1.99		2.00		2.43		0.68
Oil **	1.15±1.09	0.08±0.01	0.11±0.01	0.21±0.02	0.09±0.06	0.26±0.06	0.04±0.01	0.52±0.13
Total G+S **		1.23		0.33		0.36		0.56
Oil+cells Total G+S		3.23		2.33		2.79		1.24

3.7 Radioactive feeding experiments with putative intermediates of [6]gingerol

Reports in the literature (Denniff *et al.* 1980) suggest that phenylalanine, coumaric acid and ferulic acid are intermediates in the biosynthetic pathway leading to [6]gingerol. However, coumaric acid and ferulic acid are better precursors for [6]gingerol than phenylalanine since they are incorporated to a much greater extent (Denniff *et al.* 1980).

In this section attempts were made to investigate the biosynthetic pathway leading to [6]gingerol employing coumaric acid, ferulic acid and cinnamic acid as precursors. These molecules are all commonly used precursors of general phenylpropanoid metabolism (Gross, 1981; Sukrasno and Yeoman, 1993).

Before commencing experiments in which these radioactive precursors were fed to explants and cultures of *Z.officinale* it was necessary to synthesise these compounds.

3.7.1 Synthesis of [U-^{14}C]*p*-coumaric acid

[U-^{14}C]*p*-coumaric acid was synthesised from L-[U-^{14}C]tyrosine via a deamination reaction employing a commercial enzyme preparation of PAL (phenylalanine ammonia lyase) from *Rhodotorula glucinis.* This enzyme preparation which converts phenylalanine into cinnamic acid also possesses traces of TAL (tyrosine ammonia lyase) which converts L-tyrosine into *p*-coumaric acid.

After preliminary experiments in which the conversion of L-tyrosine to *p*-coumaric acid was optimised (see 2.10.1.1) [U-^{14}C]*p*-coumaric acid was synthesised from L-[U-^{14}C]tyrosine. 22.5μCi L-[U-^{14}C]tyrosine were dissolved in 0.5ml of distilled water, to which 125μl of 50mM Tris buffer at pH 8.5 and 0.25units of PAL were added. The reaction mixture was then incubated on a shaker at 200rpm at 30°C for 16h. 0.1ml of 6N HCl was added to stop the reaction and to ionise the remaining

amino acid to ensure the L-[U-^{14}C]tyrosine would remain in the aqueous fraction during extraction. The [U-^{14}C]*p*-coumaric acid formed was then extracted 5X with 1.5ml of EtOAc and the combined extracts dried down under vacuum. The residue was then dissolved in 1ml of HPLC grade MeOH, filtered through a 0.45μm nylon filter and stored in the refrigerator at 5°C until required. The purity of the product was checked by TLC with solvent system II which separates [U-^{14}C]*p*-coumaric acid from L-[U-^{14}C]tyrosine, which remains at the origin.

The radioactive spots on the TLC plate were first located using a TLC-RITA scan (see 2.10.3.2) then the reaction product was removed from the plate and counted in a scintillating counter (see 2.10.3.3). A conversion of *ca.* 85% was achieved.

The results presented in Fig. 3.7.1 show a RITA scan of the TLC plate in which most of the L-[U-^{14}C]tyrosine has been converted to [U-^{14}C]*p*-coumaric acid. There is also an unknown third compound.

3.7.2 Synthesis of [U-^{14}C]cinnamic acid

The method used was similar to that already described for the conversion of L-[U-^{14}C]tyrosine to [U-^{14}C]*p*-coumaric acid (see 3.7.1). [U-^{14}C]cinnamic acid was synthesised from [U-^{14}C]phenylalanine employing a commercial PAL enzyme from *Rhodotorula glucinis* which deaminates L-phenylalanine to cinnamic acid.

20μCi of [U-^{14}C]phenylalanine dissolved in 0.5ml of distilled water was added to 125μl of 50mM Tris buffer at pH 8.5 together with 0.25units of PAL and the mixture incubated on a shaker at 200rpm at 30°C for 16h. The reaction was stopped by the addition of 0.1ml of 6N HCl which also ionises the remaining amino acid and ensures it remains in the aqueous fraction during extraction. The [U-^{14}C]cinnamic acid formed was extracted 5X with 1.5ml of EtOAC. The combined extracts were dried under vacuum and the residue dissolved in 1ml of HPLC grade MeOH. This solution was then filtered through a 0.45μm nylon filter and stored in the refrigerator at 5°C

until required. The purity of the product was checked on TLC using solvent system II which separates the [U-^{14}C]cinnamic acid formed from the amino acid [U-^{14}C]phenylalanine which remains at the origin.

The radioactive spots on the TLC plate were first located by TLC-RITA scan (see 2.10.3.2), then the reaction product was removed from the plate and counted using a scintillating counter (see 2.10.3.3). A conversion of *ca.* 87.0% was achieved.

The results presented in Fig. 3.7.2 show a RITA scan of the TLC plate in which most of the [U-^{14}C]phenylalanine has been converted to [U-^{14}C]cinnamic acid.

Figure 3.7.1 A TLC-RITA scan of the reaction products of L-[U-^{14}C]tyrosine incubated with PAL for 16h, 1= *p*-coumaric acid , 2= tyrosine, 3= unknown. TLC developed in solvent system II

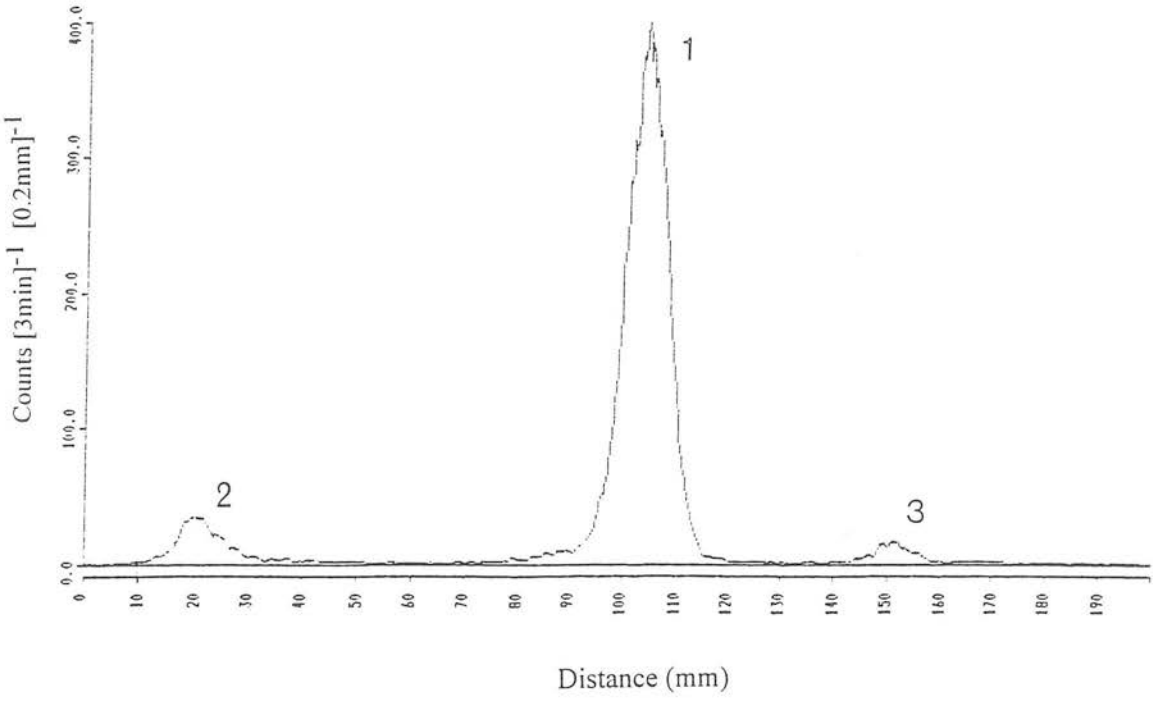
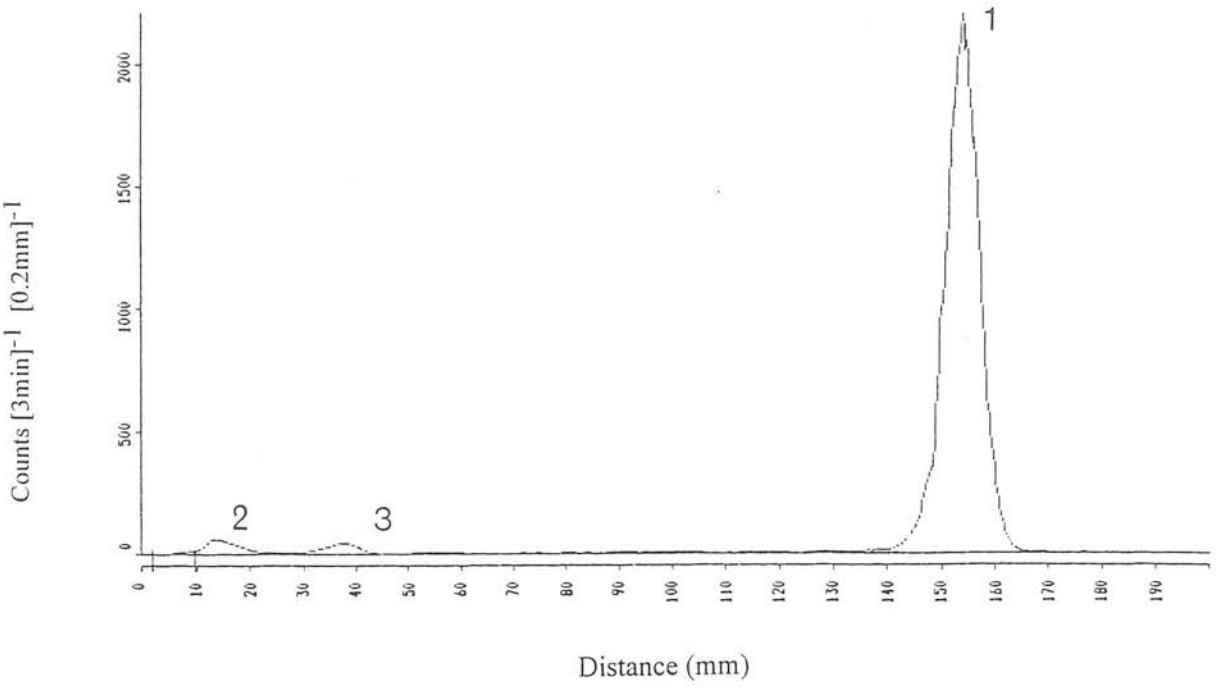


Figure 3.7.2 A TLC-RITA scan of the reaction products of [U-^{14}C]phenylalanine incubated with PAL for 16h, 1= cinnamic acid , 2= phenylalanine, 3= unknown. TLC developed in solvent system II



3.7.3 Fate of phenolic pungent principles in cultured rhizome blocks

Prior to the commencement of feeding experiments with radiotracers a study was conducted over a 14d period to assess whether the amounts of [6]gingerol and related compounds change with time in cultured rhizome blocks.

Rhizomes from mature ginger plants (*ca.* 4 months old) grown in the greenhouse (see 2.1) were washed under running tap water and the scale leaves removed with a scalpel. The rhizome was then surface sterilised in 80% (v/v) EtOH for 30sec, followed by immersion in a 20% (v/v) solution of hydrogen peroxide (30% stock solution) for 4min. The rhizomes were then transferred to a 20% (v/v) solution of sodium hypochlorite (1.5% available chlorine) containing 4-5 drops of a wetting agent (Tween-80) which was stirred by means of a magnetic stirrer for 30-35min. Finally, the rhizomes were rinsed five times with sterile distilled water to remove the residual sodium hypochlorite. After surface sterilisation the rhizomes were sliced with a scalpel to a thickness of approximately 1cm and cylinders (1x1cm length and diameter) removed with a cylindrical cork borer of 1cm in diameter, each explant weighed *ca.* 1.3g in fw.

The blocks were then placed aseptically onto pieces of sterilised moist filter paper contained in 5cm diameter Petri dishes which were double sealed with Parafilm to preserve a humid environment. The dishes were then placed in a constant temperature room at 25±2°C under standard culture conditions (see 2.2.3.1). Three replicates were harvested at d0 (immediately after the blocks were removed), and at d4, d7 and d14.

After harvesting the tissue was cut into small pieces and then ground up in a pestle and mortar with a small volume of acetone *ca.* 5ml. Once the material had been ground up, an extra 60-70ml of acetone was added to the mortar and the slurry transferred to a 100ml Erlenmeyer flask. The mixture was then extracted by stirring at room temperature for 4h, filtered as described in 2.3.1, evaporated to dryness under

vacuum, taken up in 1ml of HPLC MeOH and finally filtered through a 0.45μm Nylon filter (see 2.3.1) Quantitative analysis was performed by HPLC (see 2.6).

The results presented in Table 3.7.1 show the amounts of three PPPs ([6]gingerol, [6]shogaol and [4]shogaol) which are present in the tissue extract. It can be seen that the amounts of all three PPPs appeared to decrease slightly between d0 and d4. However, a statistical comparison of the PPPs of the d0 and d14 values were not significantly different at P=0.05, (see Table 3.7.1). Therefore, it would appear that the amount of PPPs present in the blocks does not vary with time. This means that any of these compounds labelled with a radiotracer will be diluted considerably by the high amounts of PPPs present in the tissue.

In the next section attempts were made to label these PPPs by feeding radioactive tracers to ginger rhizome blocks.

Table 3.7.1 Amounts of [6]gingerol, [6]shogaol and [4]shogaol present in the acetone extracts of rhizome blocks from d0 to d14. Each value (μg g^{-1}fw) is the mean of three replicates ± se

Time	PPPs (μg g^{-1}fw)		
	[6]gingerol	**[6]shogaol**	**[4]shogaol**
d 0	998.50±89.0^{a}	833.0±81.62^{b}	401.6±27.80^{d}
d 4	732.1±150.0^{a}	565.7±177.0^{b}	289.2±51.64^{d}
d 7	641.50±96.2^{a}	472.8±110.0^{b}	262.7±36.20^{d}
d 14	675.1±113.0^{a}	571.7±135.0^{b}	264.0±73.50^{d}

Values within a column not followed by the same letter differ significantly (P=0.05) by one way analysis of variance (t-Test).

3.8 Radioactive feeding experiments with rhizome blocks and cultures of *Z.officinale*

3.8.1 Radioactive feeding experiments with [U-^{14}C]*p*-coumaric acid using rhizome blocks

Rhizomes from mature plants (4 months old) grown in the greenhouse were surface sterilised and blocks of tissue *ca.* 1.3g in fw removed as described previously (see 3.7.3). Individual blocks of sterilised material were placed on top of sterilised moist filter paper in small Petri dishes (5.0cm in diameter). 0.48μCi of [U-^{14}C]*p*-coumaric acid, synthesised as described in 3.7.1, in 55.5μl of 50% MeOH in water was added slowly to each block in drops from a micro-syringe to allow the solution to be absorbed by the tissue. The Petri dishes were then double sealed with Parafilm and incubated under standard culture conditions until required.

Three replicates were harvested immediately after addition of the precursor and then at 24h and 48h. The tissue was extracted with acetone as described in 3.7.1. The samples dissolved in 1.5ml MeOH (HPLC grade) were filtered and retained for analysis. Quantitative determination of the PPPs was conducted by HPLC (see 2.6). The filter paper was extracted with MeOH. The extracts were analysed by TLC, using silica gel plates, developed with solvent system II and autoradiographs prepared (see 2.10.3.1) to localise the labelled compounds. In addition the radioactivity incorporated into the different fractions as well as of the radioactivity in the separated spots were counted by scintillation (see 2.10.3.3).

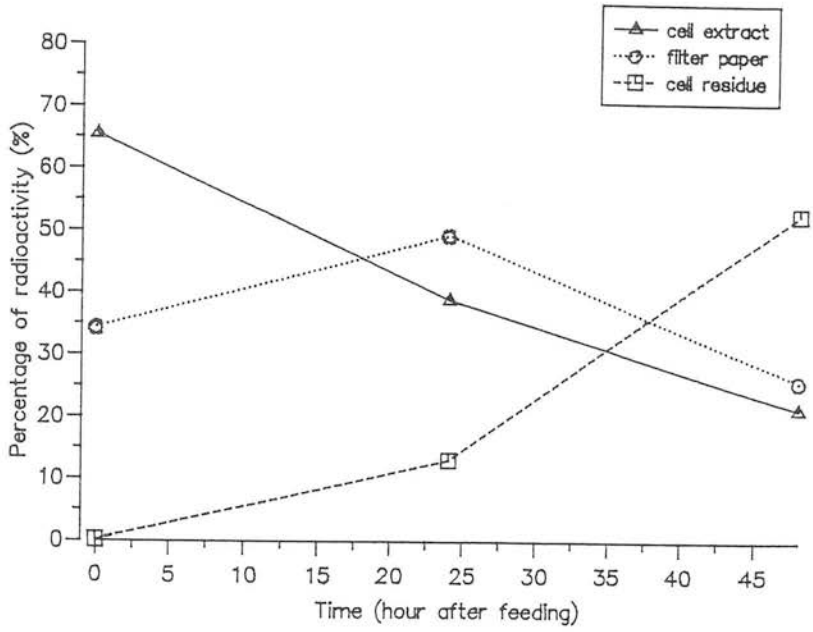
The results presented in Fig. 3.8.1 show the distribution of radioactivity within the fractions. It can be seen that at time 0 a substantial proportion of the radioactivity *ca.* 35% was present in the MeOH filter paper extract. This reached a maximum value of *ca.* 50% after 24h and approximately 25% after 48h. It would appear from this that a considerable proportion of the radioactivity was washed away and had not been absorbed by the plant tissue. It can also be seen that the radioactivity present within the cell residue was as expected vanishingly small immediately after feeding but then increased dramatically with time reaching 10% at 24h and *ca.* 54% after 48h. In contrast, the radioactivity recorded in the acetone cell extract was maximal at time 0 (65%) due presumably to the uptake of this compound by the cells prior to metabolism. This value decreased to 40% after 24h with a further decrease to 25% after 48h feeding.

Results of TLC autoradiography show at time zero a radioactive spot corresponding to [U-^{14}C]*p*-coumaric acid (R_f value 0.43) which is 44% of the total radioactivity within the acetone cell extract. There were also compound(s) at the loading point which did not run with the solvent system and contained 1.2% of the added radioactivity. However, 24h after feeding a radioactive labelled compound (compound-1) with an R_f value of 0.55 was recorded with *ca.* 1% of the radioactivity, again there was also some radioactivity left behind at the loading point (*ca.* 15%) this

could have been a mixture of conjugated phenolic(s) which did not migrate with the solvent system. At 24h the radioactivity in the [U-^{14}C]*p*-coumaric acid spot was just 4.5%, far less than that recorded at time zero. After 48h the radioactivity in [U-^{14}C]*p*-coumaric acid was lower (1.34%) while the radioactivity at the loading point was 20% (similar to that recorded after 24h). No other radioactive spots were observed at 48h.

Unfortunately none of the known PPPs ([6]gingerol, [6]shogaol or [4]shogaol) were radioactive. Thus it would appear that *p*-coumaric acid was not incorporated into the pathway leading to [6]gingerol. Most of the radioactivity was either present at the loading point or within the cell residue indicating another metabolic fate of the added compound.

Figure 3.8.1 Changes in the distribution of [^{14}C] in the different fractions after feeding rhizome blocks with 0.468μCi of [U-^{14}C]*p*-coumaric acid. Each value is the mean of three replicates



After the unexpected lack of success with feeding [U-^{14}C]*p*-coumaric acid it was decided to use [U-^{14}C]cinnamic acid, an earlier intermediate in the phenylpropanoid pathway, which might penetrate more rapidly and therefore be more effective as a precursor for [6]gingerol and related compounds.

3.8.2 Radioactive feeding experiments with [U-^{14}C]cinnamic acid using rhizome blocks

Rhizome blocks were obtained and treated in exactly the same way as described in 3.8.1. 0.35μCi of [U-^{14}C]cinnamic acid, synthesised as described in 3.7.2 dissolved in a solution of MeOH-H_2O (1:1) was administered to each block by slowly dripping the solution onto the tissue placed on top of a piece of sterilised moist filter paper contained in a 5cm Petri dish. The dishes were then sealed with Parafilm and placed under standard culture conditions as previously stated until harvested at 0h, 24h and 48h after feeding (see 3.8.1).

Tissue was extracted and the radioactivity in the different fractions and the labelled compounds located and analysed as described in 3.8.1. Quantitative determination of the PPPs in the blocks was conducted by HPLC (2.6). The results presented in Fig. 3.8.2 show the distribution of radioactivity within the fractions. It can be seen that at time 0, 30% of the radioactivity was present in the filter paper which shows that at this stage not all of the precursor was absorbed by the tissue. However, the proportion in the filter paper decreased with time, 17% and 10% after 24h and 48h respectively. The radioactivity present in the cell residue exhibited a similar trend to that observed with [U-^{14}C]*p*-coumaric acid, immediately after feeding there was no incorporation but after 24h there was *ca.* 17% and 43% after 48h. This was less than that recorded with [U-^{14}C]*p*-coumaric acid (54%). In contrast, the radioactivity present in the cell extract was maximal at time 0 (70%) and then decreased to 66% and 47% after 24h and 48h respectively.

To determine whether this putative precursor was incorporated in any of the known PPPs of ginger developed TLC plates were autoradiographed (see 2.10.3.1) and the radioactive spots scraped and counted using a liquid scintillation counter (see 2.10.3.3).

The results presented in Fig. 3.8.3 show the changes in the distribution of radioactivity for each spot with time (data for time 0 not plotted since the only radioactive spot recorded corresponded to cinnamic acid). It can be seen that several compounds were labelled. The radioactivity present at the loading point was similar at both 24h and 48h (20% of the initially added 0.35μCi). Compound-1 (R_f value of 0.13) showed a decrease in radioactivity after 48h (half the value of that at 24h, 6%). This unknown compound did not appear to be a phenolic since it did not react when the mixture ferric chloride-potassium ferricyanide was sprayed or may have been present at such a low concentration that it did not react. It can also be seen that cinnamic acid was converted to coumaric acid after 24h (compound-2, R_f value 0.38). The results presented in Fig. 3.8.3 also show that the maximum radioactivity in coumaric acid (23%) was recorded after 24h, and decreased to almost half after 48h. Compound-3 which displays the same R_f as [6]gingerol (0.46) was also labelled accounting for 2% and 0.7% of radioactivity after 24h and 48h respectively. This decline in the radioactivity is not accompanied by a reduction in the amount of [6]gingerol (data not shown) a similar result was reported in 3.7.3. Compound-4 which was located at the same R_f value as putative zingerone was also present (R_f value 0.52) and contained 5% of the radioactivity at 24h with 2.4% at 48h. Compound-5 (R_f value 0.6) was present both after 24h and 48h (1.29% and 0.65% of the radioactivity). Compound-6 with an R_f value of 0.68 corresponded to [U-^{14}C]cinnamic acid and accounted for 10% after 24h but only 1% after 48h. Finally, compound-7, probably an isomer of cinnamic acid (R_f value 0.75) was also detected after 24h (2.4%) and 48h (0.72%).

In contrast to the results from 3.8.1, when [U-^{14}C]*p*-coumaric acid was used, here the addition of [U-^{14}C]cinnamic acid labelled some of the ginger phenolics. It was also observed that after 48h most of the labelled compounds (except those at the loading point on TLC) showed a lower radioactivity level compared to that at 24h. Dilution of newly synthesised PPPs does not seem to be the case since the amount of

the PPPs present in the blocks remained constant from 0h to 48h (data not shown). These results are consistent with those presented in 3.7.3 which showed that even after 14d the level of the PPPs in rhizome blocks remained unchanged. Furthermore, the high amount of radioactivity present in the cell residue which reached a maximum after 48h is possibly due to phenolic conjugates.

Figure 3.8.2 Changes in the distribution of [^{14}C] in different fractions after feeding rhizome blocks with 0.35μCi of [U-^{14}C]cinnamic acid. Each value is the mean of three replicates

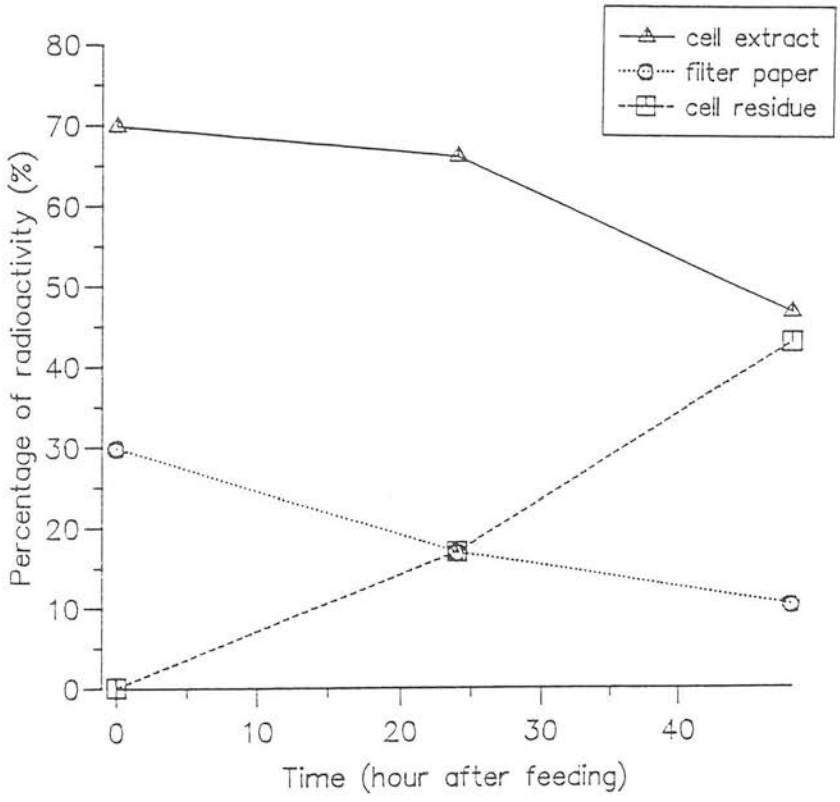
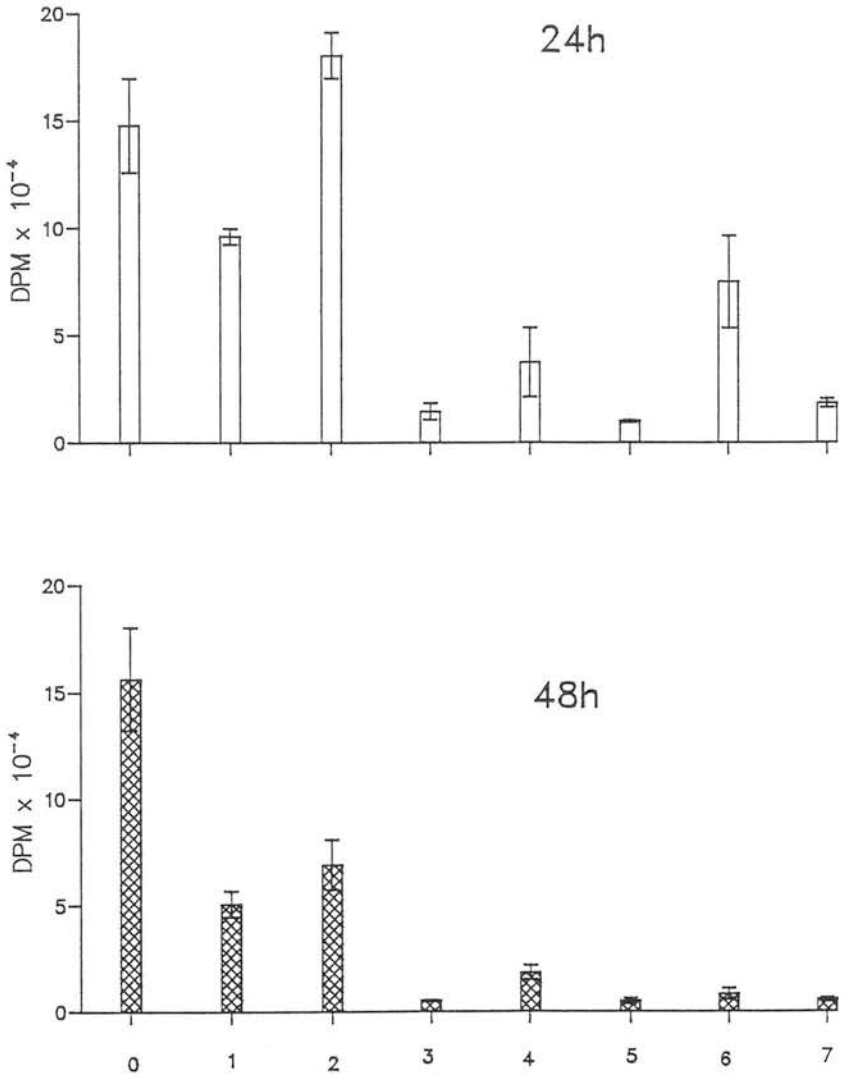


Figure 3.8.3 Changes in the distribution of [^{14}C] in different compounds in the cell extract fraction analysed by TLC with solvent system II and after autoradiography. 0= loading point, 1= unknown compound, 2= coumaric acid, 3= [6]gingerol, 4= zingerone ?, 5= unknown compound, 6= cinnamic acid, 7= cinnamic acid isomer ?. Each value is the mean of three replicates ± se



In the next subsection (3.8.3) the availability of [^{14}C-methyl] ferulic acid made it possible to evaluate the effectiveness of this intermediate to the biosynthetic pathway leading to [6]gingerol. In theory this precursor which is closer to the end product should be incorporated more efficiently (Lindsey and Yeoman, 1984a,b and Yeoman *et al.*, 1990).

3.8.3 Radioactive feeding experiments with [^{14}C-methyl]ferulic acid using rhizome blocks

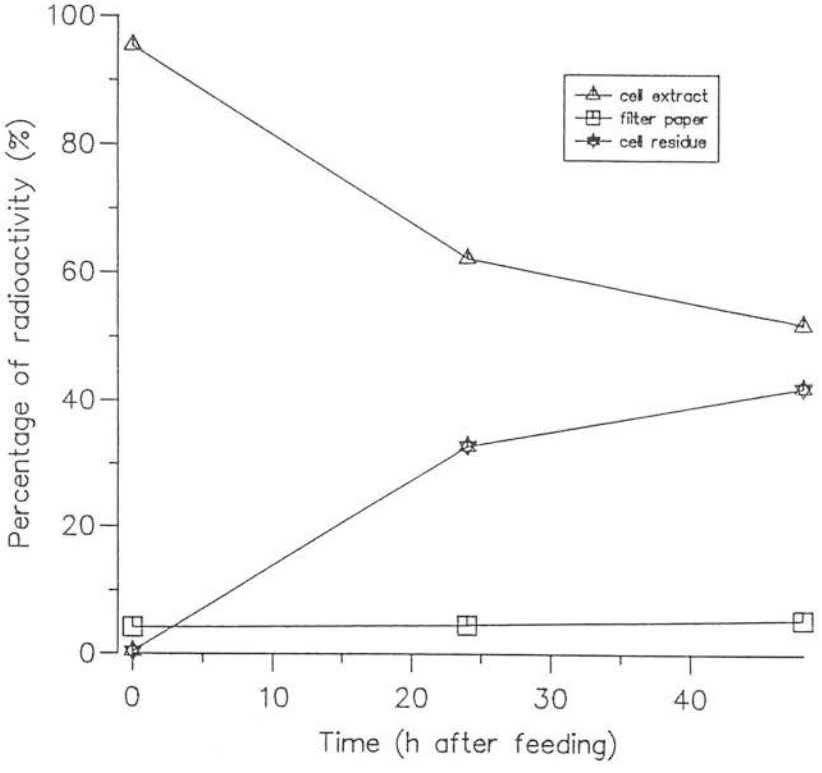
This experiment was similar to those described in 3.8.1 and 3.8.2 and was conducted with [^{14}C-methyl]ferulic acid (kindly donated by Dr. Myton). Prior to feeding the radioactive compound was purified by preparative TLC on silica-gel K-5, 250μm in thickness (Whatman) using solvent system II. [^{14}C-methyl]ferulic acid was detected by autoradiography (2.10.3.1). After autoradiography of the developed plate the silica was scraped off and extracted 5X with EtOAc. The combined fractions were evaporated under vacuum, dissolved in 1.5ml of MeOH (HPLC grade) and filtered through a 0.45μm nylon filter. The purity of the compound was then checked prior to the feeding experiments.

Rhizome blocks were obtained and treated in exactly the same way as described in 3.8.1. After purification 0.50μCi of [^{14}C-methyl]ferulic acid dissolved in a solution of MeOH-H_2O (1:1) was administered to each block by slowly dripping the solution onto the tissue placed on top of a piece of sterilised moist filter paper contained in a 5cm Petri dish. The dishes were then sealed with Parafilm and placed under standard culture conditions until harvested at 0h, 24h and 48h after feeding (see 3.8.1). The tissue was acetone extracted and the radioactivity of the different fractions and compounds localised and counted as described earlier (see 3.8.1).

The results depicted in Fig. 3.8.4 show the distribution of radioactivity within the different fractions. Contrary to the results previously obtained in 3.8.1 and 3.8.2 here the radioactivity present in the MeOH filter paper extract was constant throughout the experimental period with a maximum of just 5.5% after 48h, indicating that most of the compound was quickly absorbed by the plant tissue. Similar to the results obtained with [U-^{14}C]cinnamic acid and [U-^{14}C]*p*-coumaric acid (3.8.1 and 3.8.2) there was no radioactivity in the cell residue immediately after feeding, this then increased with time to 33% after 24h and 42% after 48h. In contrast the amount of

radioactivity present in the acetone cell extract showed the opposite pattern with the highest value immediately after feeding (95%) with 62% and 52% after 24h and 48h respectively.

Figure 3.8.4 Changes in the distribution of [^{14}C] in different fractions after feeding 0.50μCi of [^{14}C-methyl]ferulic acid to rhizome blocks. Each value is the mean of three replicates



TLC autoradiography showed that the only labelled compound present at 0h was [^{14}C-methyl]ferulic acid and accounted for 77.5% of the radioactivity. After 24h and 48h residual precursor accounted for 4.8% and 1.6% respectively similar to the results described in 3.8.1 and 3.8.2. Again substantial amounts of radioactivity were present at the loading points suggesting that conjugated compound(s) were present which did not run with the solvent system used (2% at 0h, 62.63% at 24h and 53% at 48h). After spraying the plate with a mixture of ferric chloride-potassium ferricyanide (see 2.5.2.3) these radioactive spots at the loading points did not react either indicating the presence of a very small quantity of the compound not sufficient to give a reaction or that the compounds were not phenolic. The areas on the TLC corresponding to [6]gingerol, [6]shogaol and zingerone did not contain any radioactivity.

It would appear from these results that despite the fact that [^{14}C-methyl]ferulic acid is closer to the final product ([6]gingerol) it was not incorporated into any of the known PPPs of ginger.

It has now been shown (3.8.1, 3.8.2, 3.8.3) that incorporation of ^{14}C into some of the known PPPs of ginger takes place only when [U-^{14}C]cinnamic acid was fed to rhizome tissue. Therefore it would appear that among the three putative intermediates employed only [U-^{14}C]cinnamic acid was effective. Accordingly, it was now decided to administer this compound to suspension cultures to investigate the fate of this compound and its possible incorporation into the PPPs known to accumulate in these cells during a culture cycle.

3.8.4 Radioactive feeding experiments with suspension cultures

Suspension cultures grown in medium 1/2MSB were employed in this part of the investigation. It has already been shown that these cultures accumulate [6]gingerol which may be present in the medium (see 3.5.3) or in the cells (see 3.5.4) or both. It

has also been shown that 30d cultures already in the stationary phase of growth can accumulate these PPPs suggesting a possible connection with cell differentiation.

30d suspension cultures (40ml) grown in 1/2MSB medium were fed with 48.6µl of a MeOH solution (1:1 with water) corresponding to 1µCi of [U-^{14}C]cinnamic acid (synthesised as described in 3.7.2). After feeding, the flasks were incubated on an orbital shaker under standard cultures conditions until required. Three replicates were harvested immediately after the addition of the precursor and then after 24h, 48h and 7d. The cells and medium were separated by filtration. Cells were extracted with acetone (see 2.3.2.2); and the medium with Et_2O-EtOAc (see 2.3.2.2.1). Quantitative analysis of the samples from both fractions was carried out by HPLC (see 2.6).

The radioactivity present in each of the fractions was determined using a liquid scintillation counter (see 2.10.3.3) and the results are presented in Fig. 3.8.5. It can be seen that the radioactivity present in the organic fraction of the medium reached a maximum (48%) immediately after feeding which then declined to values close to zero from d 1 to d 7. Conversely the radioactivity in the aqueous fraction of the medium showed a steady increase throughout the experimental period from 18% at time 0h to a maximum of 48% at d 7. The presence of radioactivity in this fraction may indicate that the [^{14}C] is bound to polar moieties such as sugars. The radioactivity in the cell residue was minimal at 0h (9%) and gradually increased to reach a high value (42%) after 24h which then remained constant until the end of the experiment. Finally, the radioactivity present in the cell extracts was highest at 0h and 7d (26%) and then declined slowly with time to reach 21% and 15% after 48h and 48h respectively. Quantitative analysis of the cell and medium extracts showed a lack of PPPs in the organic fraction of the liquid medium; however, detectable amounts of [6]shogaol were recorded in the cell extract although [6]gingerol was not recorded. The organic and cell extracts were then analysed on TLC using solvent system II and after autoradiography it was observed that at time 0h most of the radioactive precursor added (49%) was present in the organic fraction of the liquid medium (see

Fig. 3.8.5). The cell extract also contained cinnamic acid (R_f value 0.62) but to a much lesser extent (1%). There was also a very dark radioactive spot at the loading point on the TLC of the cell extract samples (28%). After 24h radioactive cinnamic acid was still observed in the organic fraction of the liquid medium (0.53%); however, the cell extract showed vanishingly small spots corresponding to cinnamate (0.08%) with the highest amount of [^{14}C] present at the loading point (18%), possibly from conjugated compound(s). A radioactive spot (R_f 0.28) close to [6]gingerol (R_f 0.31) was also detected (0.02%). After 48h (see Fig. 3.8.6) a small amount of radioactive cinnamate was still present in the organic fraction of the liquid medium (0.12%). Large radioactive spots were present at the loading point of cell extracts (16%) suggesting that the compound(s) present could be conjugated and therefore would not migrate with the solvent system employed. There was also a faint radioactive spot (R_f 0.4) corresponding to an unknown compound located between [6]gingerol and putative zingerone (0.02%), at this time all the radioactive cinnamate had disappeared from the cell fraction (see Fig. 3.8.6). Finally, after 7d the organic fraction of the medium did not contain any labelled compound(s). However, the cell extracts showed a similar pattern of radioactive compounds to that for previous sampling times at 0h, 24h, and 48h. There was still a very considerable amount of radioactivity at the loading point (11%); and a labelled compound located between [6]gingerol and putative zingerone (0.047%). No radioactive cinnamate or radioactive spots corresponding to the known ginger phenolics were present.

Figure 3.8.5 Changes in the distribution of [^{14}C] in the different fractions of 30d old 1/2MSB suspension cultures after feeding with 1.0μCi of [U-^{14}C]cinnamic acid. Each value is the mean of three replicates

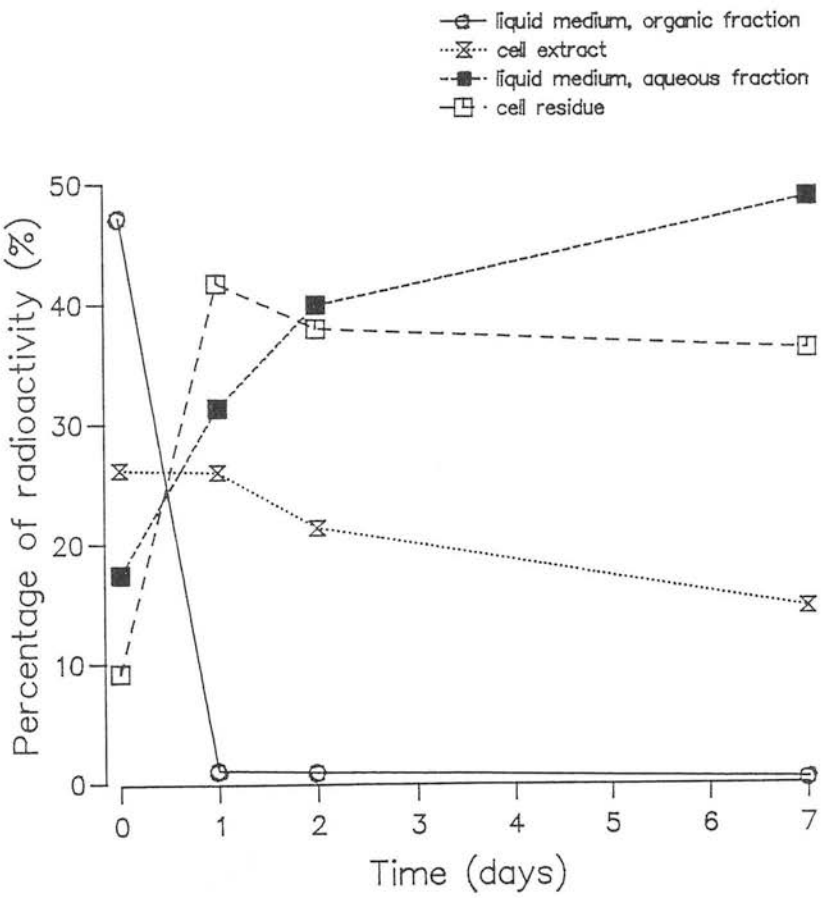
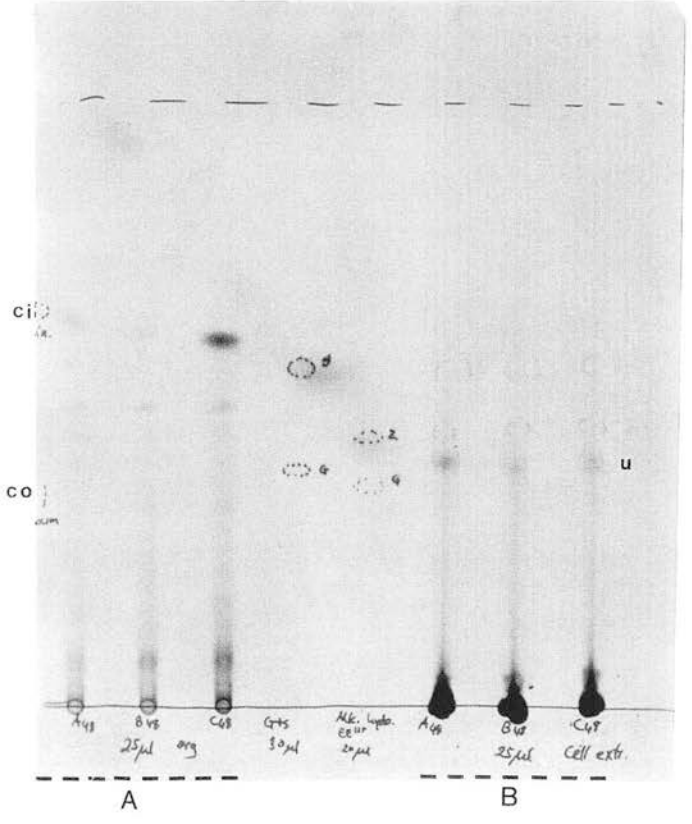


Figure 3.8.6 Autoradiograph of a TLC of samples 48h after feeding suspension cultures with 1μCi [U-^{14}C]cinnamic acid. A= three replicates of the organic fraction of the liquid medium showing radioactive cinnamate, B= three replicates of the cell extract, observe a very dark radioactive spot at the loading points and a radioactive spot corresponding to an unknown compound located between [6]gingerol and putative zingerone. None of the ginger phenolic were labelled. ci= cinnamic acid, co= coumaric acid, G= [6]gingerol, Z= zingerone ?, S= [6]shogaol, u= unknown



In view of the large amounts of radioactivity present at the loading points of the cell extracts, possibly due to the presence of conjugated compound(s), it was decided to investigate this artefact by fractionating the acetone cell extract with hexane-water. Accordingly the cell extract dissolved in 250µl of MeOH was evaporated to dryness under a stream of nitrogen and the residue taken up in 5ml of water to dissolve any sugar conjugates. 5ml of hexane was added to the vial to remove the remaining matter (which did not dissolve completely with water). The two fractions were mixed up in a separating funnel and LLE performed 3x. The combined organic fractions were evaporated to dryness under vacuum and finally taken up in 250µl of MeOH. The samples from the organic fraction were then analysed by TLC and autoradiography as described previously.

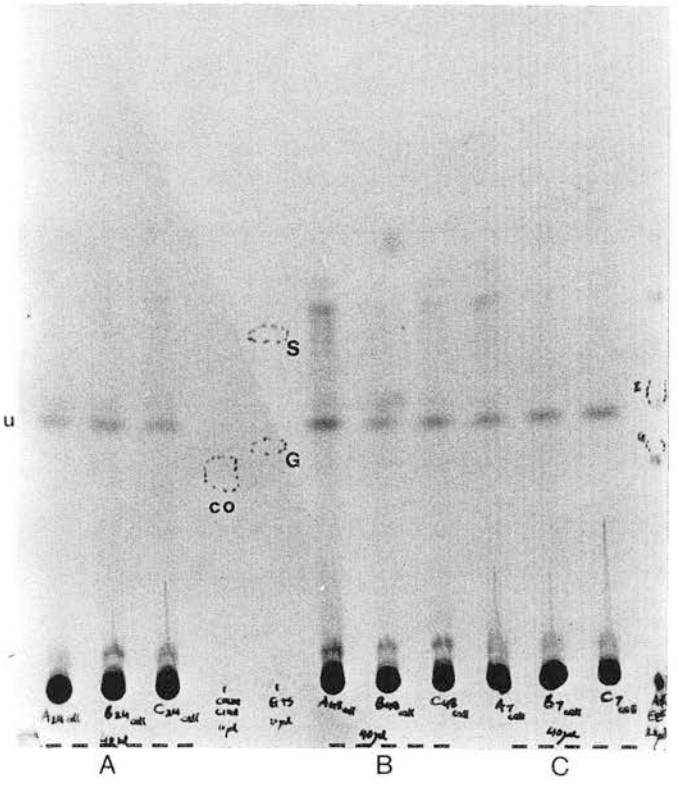
Radioactivity in the samples from the hexane fraction and identification of the labelled compound(s) was conducted on TLC after autoradiography. It was found that after fractionation a radioactive spot located between [6]gingerol and putative zingerone was present (see Fig. 3.8.7), probably the same compound detected in samples 24h, 48h, and 7d prior to fractionation (see Fig. 3.8.6). The radioactivity of these compounds showed a very similar proportion (0.04%, 0.05%, 0.047%) after 24h, 48h, 7d respectively; moreover, after fractionation none of the known PPPs appeared labelled in the hexane. Therefore, it would appear that the addition of [U-^{14}C]cinnamic acid to 30d suspension cultures did not label any of the known PPPs although quantitative analysis detected [6]shogaol, furthermore, a large proportion of radioactivity was observed at the loading points on TLC which perhaps might contain conjugate(s).

(3.6.3)

It has already been shown in this thesis that PPPs can be trapped in sunflower oil added to suspension cultures one possibility is that newly synthesised PPPs will be released or become conjugated in the medium. In order to increase the amount of radioactivity into the PPPs it was decided to label suspension cultures with [U-^{14}C]cinnamic acid in the presence of sunflower oil to trap newly synthesised PPPs.

Accordingly in the next sub-section [U-^{14}C]cinnamic acid was fed to suspension cultures supplemented with 5ml of sunflower oil.

Figure 3.8.7 Autoradiograph of a TLC of the 24h, 48h and 7d samples after fractionation with hexane-water of the cell extract of suspension cultures fed with 1μCi [U-^{14}C]cinnamic acid. Observe the same unknown compound located between [6]gingerol and putative zingerone also observed prior to fractionation. A= three replicates of the cell extract of 24h labelling after fractionation, B= three replicates of the cell extract of 48h labelling after fractionation, C= three replicates of the cell extract of 7d labelling after fractionation. None of the ginger phenolic were labelled. ci= cinnamic acid, co= coumaric acid, G= [6]gingerol, Z= zingerone ?, S= [6]shogaol, u= unknown



3.8.5 Radioactive feeding experiments with suspension cultures supplemented with sunflower oil

It has been established that [6]gingerol the main PPP of ginger occurs together with a series of metabolites (flavonoid-like compounds, curcumins and possibly other PPPs) in yellow idioblasts distributed in tissue of the rhizome. These compounds are found together with oil and the components of the essential oil of the spice (3.2). In earlier experiments it was shown that when sunflower oil was added to suspended cells the PPPs accumulated within the oil (3.6.3) mimicking the situation observed in the rhizome where the PPPs were found in the oil storage cells (3.2). It is therefore possible that freshly synthesised radioactive PPPs will largely escape conjugation and accumulate in the sunflower oil. This might also be expected to enhance synthesis by removing the product.

Accordingly 30d old suspension cultures grown in 1/2MSB (40ml) were fed with [U-^{14}C]cinnamic acid in the presence of sunflower oil. Two treatments (three replicates each) were used: cultures supplemented with 0.94μCi [U-^{14}C]cinnamic acid (control) and cultures supplemented with 0.94μCi [U-^{14}C]cinnamic acid together with 5ml of sterilised sunflower oil (treatment). The oil was added *ca.* 15min after feeding the radioactive precursor. Cultures were then placed on an orbital shaker under standard culture condition and harvested after 24h.

The oil was separated from the culture as described in 3.6.3, extracted 3X with MeOH and the samples prepared for analysis (TLC and HPLC). The medium and cells were separated by filtration, extracted with EtOAc-Et_2O, and acetone respectively (see 2.3.2.2 and 2.3.2.2.1) and the samples prepared for analysis. The radioactivity present in all the fractions was determined by liquid scintillation counting (see 2.10.3.3) After TLC, using solvent system II, the compounds were detected by autoradiography (see 2.10.3.1). The distribution of radioactivity [^{14}C] in the five fractions after 24h is shown in Fig. 3.8.8. It can be seen that the amount of

[^{14}C] in the organic fraction of the medium was very low (1.3x10^4 DPM) and was similar for both, cultures with [U-^{14}C]cinnamic acid alone (control) and those with [U-^{14}C]cinnamic acid and sunflower oil (treatment). Moreover, the radioactivity present in the aqueous fraction of the liquid medium was significantly higher in cultures without oil, whilst the amount of radioactivity present in the cell extract was similar for cultures with or without oil (2x10^4 DPM). The highest amount of [^{14}C] was present in the cell residue, 5x10^5 DPM cultures without oil and 4.3x10^5 DPM with oil. The MeOH oil extract was also radioactive (*ca.* 4.3x10^4 DPM) which indicates the presence of radioactive non-polar compound(s). Finally, the amount of [^{14}C] in the remaining oil fraction after extraction was vanishingly small (330 DPM) which shows the high extraction efficiency with MeOH.

Autoradiography of the samples from control and treatment cultures showed the presence of several labelled compounds (see Figs. 3.8.9; 3.8.10). In the organic fraction of the medium after LLE, the only labelled compound recorded was [U-^{14}C]cinnamic acid for both cultures with oil or without oil. Samples from the cell extracts of cultures with or without oil showed three main labelled spots (see Table 3.8.1). A very dark spot at the loading point on the TLC was observed; suggesting the presence of conjugated compound(s) which did not run with the solvent system used. The amount of [^{14}C] at the loading points was similar for control (18.5x10^3 DPM) and treatment (17.4x10^3 DPM). A spot with the same R_f as [6]gingerol (0.43) was recorded in both control and treatment with similar amounts of activity, control (3.5x10^3 DPM) treatment (3.4x10^3 DPM). The last labelled compound recorded in the cell extract was residual cinnamic acid which displayed a similar activity for control (0.85x10^3 DPM) and treatment (1.3x10^3 DPM). Finally the MeOH oil extract showed the presence of a conspicuous labelled spot corresponding to cinnamic acid with a very high activity 2.3x10^4 DPM indicating that the oil fraction has trapped some of the added [U-^{14}C]cinnamic acid. A second labelled spot of an unknown compound with an R_f value close to that of coumaric acid was also detected with an

activity of $1x10^3$ DPM (see Fig. 3.8.10). Therefore it would appears that the addition of oil to cultures prevents the availability of substantial amounts of radioactive cinnamate to the cells.

Table 3.8.1 Amount of radioactivity present in the different labelled compounds of the cell extract of 30d suspended cells incubated with [U-^{14}C]cinnamic acid (control) and [U-^{14}C]cinnamic acid together with 5ml sunflower oil (treatment). Each value is the mean of three replicates

Compound	**Activity (DPM)**	
	Control (no oil)	**Treatment (oil)**
Loading point	$18.5x10^3$	$17.5x10^3$
[6]gingerol	$3.50x10^3$	$3.40x10^3$
cinnamic acid	$0.85x10^3$	$1.3x10^3$

It would appear, in contrast to the results presented in 3.8.4, that incorporation of [^{14}C] into [6]gingerol has occurred in both control and treatment which would confirm that [U-^{14}C]cinnamic acid is an effective intermediate in the biosynthetic pathway leading to [6]gingerol. Also the addition of sunflower oil did not trap any [6]gingerol or [6]shogaol (data not shown) as reported previously in 3.6.3 perhaps indicating that the oil fraction was not present long enough to activate the release and accumulation of these compounds in the oil fraction. Furthermore, a substantial amount of radioactivity was present in the aqueous fraction which may suggest the formation of conjugate(s), also a lower amount of radioactivity was recorded in the aqueous fraction of treatment cultures (cultures with added oil) perhaps indicating

that the sunflower oil added to cultures lessens the glycosylation which seems to take place.

Figure 3.8.8 Changes in the distribution of [^{14}C] in six different fractions following extraction after 24h feeding 30d 1/2MSB suspension cultures with 0.94μCi of [U^{14}C]cinnamic acid, three replicates were supplemented with 5ml sterilised sunflower oil. A=organic fraction LLE of medium, B=aqueous fraction LLE of medium, C=acetone cell extract, D=cell residue, E=MeOH oil extract, F=remaining oil. Each value is the mean of three replicates ± se

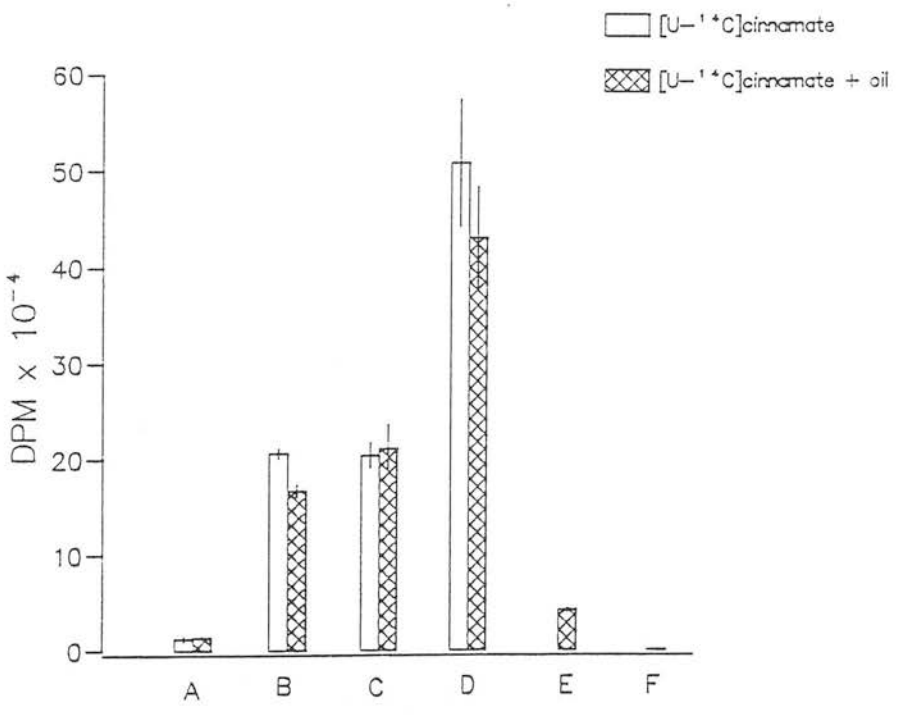


Figure 3.8.9 Autoradiograph of a TLC of the liquid medium organic fraction and cell extract samples 24h after feeding suspension cultures with 0.94μCi [U-^{14}C]cinnamic acid (control). A= three replicates of the organic fraction of the liquid medium observe a very dark spot for one of the replicates corresponding to cinnamic acid, B= three replicates of the cell extract showing three main spots: a very dark radioactive spot at the loading points and a radioactive compound with the same R_f as [6]gingerol and some spots corresponding to cinnamic acid still present after 24h. ci= cinnamic acid, co= coumaric acid, G= [6]gingerol, Z= zingerone ?, S_1= [4]shogaol S_2= [6]shogaol, u= unknown

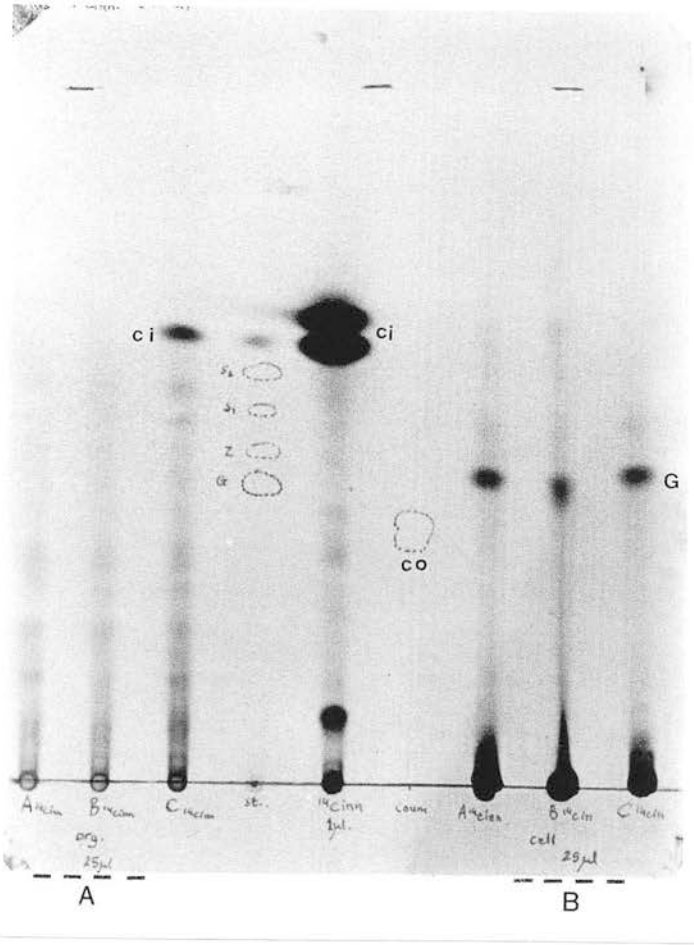
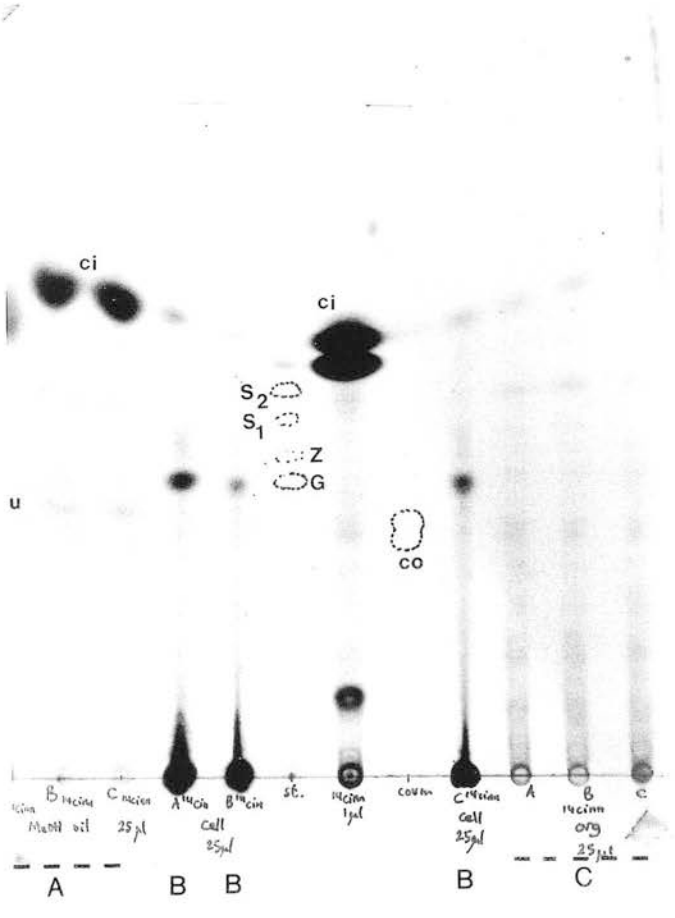


Figure 3.8.10 Autoradiograph of a TLC of samples of the organic fraction of the liquid medium, cell extract and MeOH oil extract 24h after feeding suspension cultures with 0.94μCi [U-^{14}C]cinnamic acid supplemented with 5ml of sterilised sunflower oil (treatment). An edge effect is observed which did not give a straight solvent front altering the R_f value of the spots which migrated furthest which correspond to cinnamic acid. A= three replicates of the MeOH oil extract displaying a conspicuous spot for cinnamic acid together with an unknown compound close to coumaric acid, B= three replicates of the cell extract showing three main spots: loading points, a radioactive compound with the same R_f value as [6]gingerol, cinnamic acid was also present, C= three replicates of the organic fraction of the liquid medium showing cinnamic acid. ci= cinnamic acid, co= coumaric acid, G= [6]gingerol, Z= zingerone ?, S_1= [4]shogaol S_2= [6]shogaol, u= unknown



CHAPTER 4

DISCUSSION

Studies on the production of secondary metabolites by cell cultures have demonstrated the existence of two main trends in the accumulation of secondary compounds: 1) accumulation takes place when cultures approach or enter the stationary phase of growth where there is a downturn in growth. In this case the cultures appear to have become biochemically specialised to synthesise and accumulate specific compounds and there is an inverse relationship between growth and secondary metabolite yield (Lindsey and Yeoman, 1985b; Collin, 1987);
2) accumulation takes place during different phases of growth (Noguchi and Sankawa, 1982; Hirose *et al.*, 1990; Sakamoto *et al.*, 1994). Here the increase in a particular metabolite parallels the accumulation of biomass.

In this discussion the production of the phenolic pungent principles [PPPs] by *Z.officinale* is considered, in a range of cultures, in relation to growth, as well as cells and morphological differentiation.

This discussion is divided into five sections. In the first the distribution and cellular localisation of PPPs in the plant are studied. The second section deals with the establishment of a range of cultures to enable the investigation of the production and changes in the level of these compounds under different culture conditions. In the third a consideration is made of the attempts to improve a suspension culture system to produce a higher yield of PPPs. The metabolic fate of [6]gingerol administered to suspension cultures is discussed in section four. Finally, section five assesses the fate of putative radioactive intermediates to the PPPs and their usefulness as a means of studying the metabolic pathway leading to [6]gingerol. Throughout this discussion attempts are made to draw useful comparisons between the results described in this thesis and those obtained on the production of capsaicin, the main pungent principle of the chilli plant which exhibits chemical similarities with [6]gingerol.

4.1 Distribution and cellular localisation of [6]gingerol

There are a number of reports in the literature which show that PPPs are present in the rhizome of the ginger plant. There is also evidence of the presence of oil cells in the rhizome (Winton and Winton, 1939; Govindarajan, 1982; Mangalakumari *et al.* 1984). The oil cells contain a range of constituents which give the fragrance and flavour of this spice (Lewis *et al.* 1972; Govindarajan, 1982ab; Mc Leod and Pieris, 1984). However, the distribution of these cells containing the essential oil is not limited to the rhizome as these also occur in the aerial parts of the plant mainly in the leaves (Holttum, 1950; Tomlinson, 1956 and 1962). There are however, no detailed studies on the PPP content of the oil cells present in the aerial parts of the plant which certainly contain a variety of fragrance constituents.

The results presented in this thesis clearly demonstrate that the PPPs occur mainly in the rhizome of the plant and to a much lesser extent in the adventitious roots. There is however, a total absence of these compounds in the aerial parts (pseudo-stems and leaves) (see 3.1). These results are partially consistent with those of Winton and Winton, (1939); Govindarajan, (1982ab) and Mangalakumari *et al.* (1984) who have reported the presence of these phenolic compounds only in the rhizome and not in the aerial parts of the plants, although no measurements were made on adventitious roots. In view of this distribution it would appear that the PPPs certainly accumulate in the underground parts of the plant but the absence, even of traces, of PPPs in the aerial parts would suggest they are probably synthesised in the rhizome and roots and not transported from elsewhere or that some steps of the biosynthetic pathway take place elsewhere but end in the underground parts.

Cursory examination of rhizome sections reveals the presence of yellow cells distributed randomly throughout the tissues (Mangalakumari *et al.*, 1984). In this study it was shown that these cells contain a variety of compounds including PPPs, flavonoids, possibly curcumin derivatives together with myriad constituents of the

essential oil, together with oil (see 3.2 and Zarate and Yeoman, 1994). In this instance the toxicity of non-polar phenolics, such as the PPPs, to the plant cells is avoided as they are stored in lipid material (Brown, 1981). These results contrast with the earlier findings of Mangalakumari *et al.* (1984) who reported the existence of two cell types in the rhizome one containing the pungent phenolics which are yellow in colour and another containing components of the essential oil with colourless shining oil contents; however, their results were inconclusive and some of the evidence presented was circumstantial.

Obtaining precise data on the cellular distribution of particular substances in plants is very difficult unless specific staining procedures are available for the compounds. In this instance it is possible to be certain that the cells contain storage lipid and a range of phenols, and particular pigmented materials such as flavonoids; however, in the absence of specific reagents for PPPs another approach was necessary. This was to attempt to establish a correlation between the amount of the particular compound in the tissue with the number of cells thought to contain that compound. Using this approach (see 3.2) a positive correlation was obtained between the number of pigmented cells and the amount of [6]gingerol (and possibly the other phenolics) present. It was also established using microspectrophotometry that the yellow colour in these cells was not due to [6]gingerol but to flavonoid-like compounds possibly curcumin (Harvey, 1981; Tonnensen and Karlsen, 1983), a pigment present in the rhizome of turmeric (*Curcuma domestica* L.) a plant of the same family. It was also observed that the intensity of the yellow colour varied between cells suggesting different amounts of pigment and, bearing in mind the established positive correlation, perhaps more [6]gingerol in the brighter yellow cells might be expected. This is contrary to the results of Sukrasno and Yeoman (1993) who found a decrease in flavonoids as capsaicin (the main pungent principle of the chilli fruit and chemically close to [6]gingerol) synthesis commenced with the lowest level recorded when capsaicin accumulation was at a maximum. These authors

suggest that the decrease in flavonoid which parallels the onset of capsaicin synthesis is not due to direct competition for common precursors since the two groups of compounds (capsaicinoids and flavonoids) appear to be synthesised in different compartments of the fruit.

The *in vivo* function of the PPPs in ginger is not known. It has been reported that phenolic compounds react with protein and thus make them toxic to certain microorganisms or animals by inhibiting their growth or acting as feeding deterrents giving unpleasant sensations to the taste buds of the animals (Grayer, 1989). Considering that the main phenolics of the ginger plant are located exclusively in the underground structures (rhizome and adventitious roots) it would appear unlikely that the presence of these compounds would deter grazing of the aerial parts although it might deter underground predators. However, the presence of aromatic fragrance compounds in the aerial parts of the plants could provide an effective defence mechanism (Fulder, 1993).

4.2 Establishment of cultures for studies on the production of phenolic pungent principles

The techniques of cell and tissue culture have enabled us to increase our knowledge in many areas of plant biology such as totipotency, cell and tissue differentiation, cell division, cell nutrition, plant propagation, genetic manipulation as well as plant metabolism. Particular attention has been paid to the investigation of secondary metabolism dealing with the synthesis and accumulation of commercially valuable secondary products. Such studies have contributed to our understanding of the control of specific aspects of cellular metabolism and pathways involved in the synthesis of secondary metabolites. This technique provides a number of advantages over the intact plant, for example the use of a sterile system prevents interference from microorganisms; cell and tissue culture systems can also be subjected to more

precise experimental control. Also the use of these systems can reduce the complexities in the intact plant and permit the localisation of particular reactions. Also the growing plant requires a more prolonged growth period and more physical space.

In order to study the production of PPPs it was necessary to set up a range of *in vitro* systems. Most of the research carried out so far on the culture of ginger has been specifically conducted for crop improvement via micropropagation methods designed to eliminate disease and to obtain disease-free clones (Hosoki and Sagawa, 1977; De Lange *et al.* 1987; Ilahi and Jabeen, 1987; Bhagyalakshmi and Singh, 1988; Ikeda and Tanabe, 1989; Balachandran *et al.* 1990; Nirmal-Babu *et al.* 1992). There is little information on the establishment of callus and suspension cultures which are necessary for the study of secondary metabolism (Sakamura *et al.* 1986; Charlwood *et al.* 1988) and those that exist have been concerned solely with the fragrant constituents of the essential oil and not with PPPs.

4.2.1 Establishment of callus cultures and changes in the amounts of phenolic pungent principles

It is well established (White, 1943; Dougall, 1980 and Reinert and Yeoman, 1982) that in order to induce callus, sterilised explants have to be placed in contact with a nutrient medium supplemented with a suitable combination of PGRs, usually an auxin and a cytokinin. The successful initiation of a culture is achieved empirically after repetitive testing of different media (Meins, 1986). Here, the importance of the genotype of the plant, in achieving viable cultures and use of the appropriate developmental stage at which the explant is taken, cannot be over-emphasised (Hussey, 1986). However, despite much research, the mechanisms involved in responding to different factors such as combinations of PGRs, nutrient balance, and culture conditions, are still little understood. It is however generally accepted that a

high level of an auxin, particularly the synthetic compound 2,4-D, together with a low level of a cytokinin is required for callus induction. Reports in the literature suggest that monocotyledonous species are generally more difficult to put into culture than dicotyledons (Cresswell, 1991). The much higher auxin requirement for the induction of callus with monocotyledonous species suggests that monocotyledons differ from dicotyledons. Also, because of the morphology of many monocotyledonous species, it is difficult to obtain a suitable piece of homogeneous tissue, unlike dicotyledons, where pith and cambium explants provide a uniform and homogeneous source of material for callus induction (Yamada, 1977).

In this investigation the first attempts to produce callus employed explants taken from the medulla and cortex of mature rhizomes, these were not successful (3.4.1) perhaps suggesting a developmental inadequacy (Hussey, 1986). There was also a very high incidence of contamination despite rigorous surface sterilisation pointing to the presence of endogenous contaminants. However, when 10-15d old emerging axillary buds from rhizomes were employed, callus formation was induced successfully, perhaps due to the presence of an endogenous pool of PGRs present in the active growing buds. This is consistent with the views of Yeoman and McLeod (1977) who have stated that the response of an explant in culture depends to some extent on the endogenous growth substances present in the explant at the time of excision.

Of the range of media tested two, SHB and 1/2MSB, appeared to induce callus and sustain growth (see 3.4.1). Although several reports in the literature have suggested that a high ratio of auxin/cytokinin is necessary for the successful induction of callus in monocotyledonous species (Schenk and Hildebrandt, 1972; Yamada, 1977) here the two successful media contained a lower amount of the synthetic auxin, 2,4-D, than the other media. This would suggest that a high level of 2,4-D is perhaps toxic to ginger. It was also observed that the overall increase in

biomass was very small with both media. This appears to be a general feature of ginger cultures as suspension cultures also showed slow and prolonged growth.

The changes in yellow cell numbers during callus development from explants should be viewed with extreme caution, as the number of pigmented cells per section may not be a true representation of the position within the developing callus. Technical difficulties prevented an estimation of the total number of yellow cells in each developing explant and therefore the choice of a representative sample within each individual is far from ideal. There is no doubt that within the chosen population there is a decline in the number of yellow cells and therefore a tentative interpretation of these data would suggest no correlation between PPP content per explant and the number of pigmented cells. However, this conflicts with the positive correlation established in the appropriate underground parts of the intact plant.

One clear fact which emerged from the determination of PPPs during callus development when PPP content was expressed per g fw was a steady decline in both [6]gingerol and [6]shogaol whatever medium was used. However, when the data were plotted on a per explant basis the picture was quite different with the amount of [6]shogaol remaining constant with both media whilst [6]gingerol stayed more or less constant in the medium without ammonia and increased substantially with the medium with ammonia (see Table 4.1). These data would suggest that the PPP content in the developing callus either remains constant or increases depending on the medium so that synthesis always keeps pace with degradation or exceeds it, or no degradation of the PPPs takes place. Another fact which would further affect the interpretation of these data is whether any of the PPPs are released to the agar medium. No determination were made on the medium again because of technical difficulties and for this reason much of the subsequent research on PPP production was performed with liquid cultures.

Table 4.1 A comparison of the major differences between the composition of Murashige and Skoog (1962) (MS) and Schenk and Hildebrandt (1972) (SH) medium.

Constituents	**Concentration in media (mg l^{-1})**	
	MS	**SH**
NH_4NO_3	1650	-
KNO_3	1900	2500
$NH_4H_2PO_4$	-	300
Inositol	100	1000
Casein Hydrolysate	1	-
Glycine	2	-

Although it is difficult to establish with certainty any quantitative picture of the decline in the number of yellow cells during callus development it is likely that the proportion of pigmented cells within a rapidly dividing population decreases markedly. Therefore, the fewer coloured cells present would appear to contain higher amounts of [6]gingerol which contrasts with the findings of Hall and Yeoman (1987) who suggested that variation in the accumulation of anthocyanins in *Catharanthus roseus* cultures was primarily due to differing proportions of pigmented cells rather than mean intracellular anthocyanin concentration within the cells. Also the microspectrophotometric measurements of pigmented cells from the ginger rhizome (Fig. 3.2.3a) show conspicuous differences in IOD (integrated optical density) values between cells which could support the possibility that the mean intracellular amounts of pigment and probably of PPPs vary within the cells; furthermore, the remaining yellow cells could have undergone cell expansion which would enable a higher storage potential.

It is still not known where the PPPs are synthesised, however, it has been established that these compounds are stored within the yellow cells present in tissues of the rhizome, adventitious roots and probably in cultured cells. Therefore it would appear that conditions which encourage differentiation within the cell population might promote accumulation of PPPs.

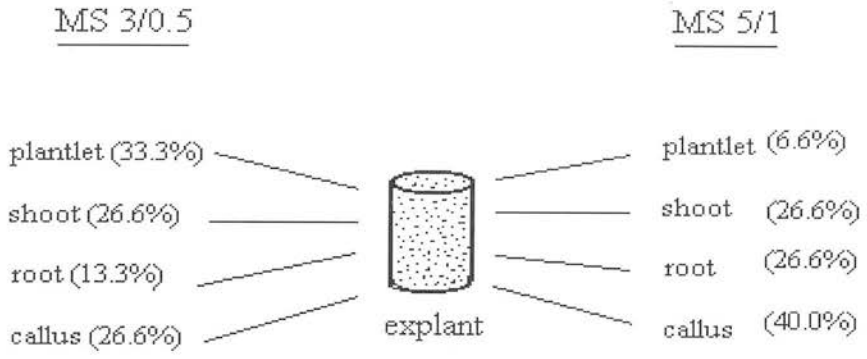
4.2.2 Induction of plant regeneration and changes in the amounts of phenolic pungent principles

It was argued in the previous sub-section that there was a decline in the number of pigmented cells as callus was formed and that the amount of [6]gingerol and [6]shogaol per explant did not keep up with the increase in biomass. This could suggest that the biosynthetic mechanisms could still be operative and that some biochemical differentiation has occurred. Therefore the promotion of differentiation by the regeneration of organs or plants, from the same source of explants (10-15d old emerging axillary buds) might be expected to promote cell differentiation and therefore PPP production.

It was demonstrated, that a range of differentiated structures could be produced from explants of the same type used to induce callus, by using different media (MS supplemented with $5mgl^{-1}$ BAP; $1mgl^{-1}$ NAA and MS supplemented with $3mgl^{-1}$ BAP; $0.5mgl^{-1}$ NAA). Explants cultured on MS 3/0.5 achieved a much higher amount of regeneration than explants on MS 5/1 (see diagram), which produced more roots and shoots than plantlets and a large proportion of callus.

Diagram

(Effect of culture medium on the induction of morphogenetic structures and plantlets from explants of *Z.officinale*. Proportion of explants undergoing morphogenesis).



Bearing in mind the limitations of the data already explained, it would appear that, even in cultures with roots and shoots, the number of pigmented cells declined, as observed with callus. Such a decrease was not unexpected, as shoots do not contain yellow cells. Furthermore, explants, which produced mainly roots, also showed a reduction in the number of coloured cells, but again, this is not surprising, since it has been shown (3.2) that the number of yellow cells present in mature adventitious roots was very low compared to rhizome tissue. However, the amount of [6]gingerol per explant was observed to rise substantially on MS 3/0.5 whereas the amount of [6]gingerol which also accumulated on MS 5/1 to some extent, during the early part of the culture cycle, was lower. This difference could be explained by the lower amount of regeneration obtained with the latter medium.

It would appear that regeneration of morphological structures is accompanied by an increase in PPPs confirming some relationship between differentiation and product yield; however, as with explants producing callus PPP production is

outpaced by increase in biomass. Again as for developing callus fewer pigmented cells would appear to be associated with higher amounts of PPPs.

In particular the mini-rhizomes which formed after transferring regenerated plantlets to a medium lacking PGRs showed substantial amounts of [6]gingerol, [6]shogaol and other phenolics (see Fig. 3.5.9c). Here, the number of yellow cells in sections was higher than in callus and regenerated plant tissue, prior to the appearance of mini-rhizomes (see Table 4.2)

Table 4.2 Amount of [6]gingerol and number of yellow cells in different tissues

Plant Material	**No. pigmented cells section^{-1}**	**[6]gingerol (μg explant^{-1})**
Rhizome	29.60	27.10
Mini-rhizome	6.10	6.58
Adventitious Roots	0.37	2.16
Callus	1.32	1.22
Regenerating explants	4.72	2.29

Therefore it would appear that the presence of a rhizome ensures the substantial accumulation of PPPs. Similar results have been reported for the production of artemisinin in cultures of *Artemisia annua* (He *et al.*, 1983) where the accumulation of artemisinin was greatly increased over callus when shoots began to form, but established axenic root cultures did not produce this compound (Woerdenbag *et al.*, 1993). These results show that specific structures are necessary for high product yield similar to the results obtained with ginger.

4.2.3 Establishment of suspension cultures and changes in the amounts of phenolic pungent principles

Suspension cultures like callus are poorly differentiated but unlike callus they are easier to manipulate and therefore provide a more amenable system for biochemical studies. The suspension cultures established in this study displayed a growth cycle characterised by a sigmoidal curve where different phases of growth could be distinguished *i.e.* lag phase, growth phase and stationary phase and these phases were accompanied by specific metabolic changes and are consistent with the results of Lindsey and Yeoman (1985b). Examples in the literature report that in suspension cultures the accumulation of secondary compounds occurs mainly when growth has ceased and cell differentiation has begun (Yeoman *et al.*, 1982). However, there are also some reports which show the accumulation of secondary metabolites during active growth; for example the accumulation of anthocyanins in cultures of *Aralia cordata* (Sakamoto *et al.*, 1994) and betacyanins in suspension cultures of *Phytolacca americana* (Hirose *et al.*, 1990). Occasionally these compounds may accumulate during the lag phase *eg.* accumulation of germichrysone in cultures of *Cassia torosa* (Noguchi and Sankawa, 1982) and carotenoid production in cultures of *Bixa orellana* (Boyd, 1991)

Suspension cultures of *Z.officinale* were established in 1/2MSB medium containing half strength MS basal salts supplemented with $1mgl^{-1}$ 2,4-D and $0.5mg\ l^{-1}$BAP with an inoculum size of 0.5g of filtered cells, an important parameter to sustain growth as reported by Gamborg and Shyluk(1981). The size of the inoculum also determined the length of the lag phase (see Fig. 3.4.3a-b) for example when an inoculum size of 1g was used there was no lag phase and the cultures entered active growth immediately but the increase in total biomass was the same. These suspension cultures grew slowly with a culture cycle of 35d and an overall increase in biomass from an inoculum of 0.5g to just 3-4g fw $flask^{-1}$.

One of the possible reasons for this slow growth was the rapid fall in pH after inoculation. Heller (1965) also reported a marked decrease in the growth of cultured tomato roots when the pH rose to 6.5. The sharp fall in pH which produced an unsatisfactory environment for the growth of cultures was probably due to changes in the ionic balance in the medium (Veliky and Rose, 1973 and Veliky, 1977).

In experiments with *Z.officinale* suspension cultures grown in 1/2MSB medium [6]gingerol accumulated and was released into the medium at d 30-40 when the cultures had reached the onset of the stationary phase showing that the accumulation of this compound is inversely proportional to growth which is consistent with the suggestion of Yeoman *et al.* (1982, 1990) and Collin (1987). However, the pattern of accumulation varied and some partitioning existed between cells and medium. It would appear that transport mechanisms may be affected, possibly through specific carriers. Intracellular and extracellular pH has also been said to influence product release (Parr *et al.* 1987) and in ginger cultures the pH was shown to vary throughout the growth cycle.

4.3 Changes to the composition of the culture medium to improve product yield

4.3.1 Effects of medium composition including the addition of glutamine

Attempts made to stabilise the pH of the medium by the addition of glutamine to MS and SH based medium were unsuccessful and the pH remained variable. A possible explanation for this variable pH is the balance of residual cations and anions left in the medium as nitrogen utilisation and growth proceeded, as well as the release of alkaline substances into the medium (Veliky and Rose, 1973). However, the addition of glutamine failed to stabilise the pH. Moreover, the pattern of PPP accumulation was also altered (see Table 4.3) with a reduction in the amount of [6]gingerol in the medium of 1/2MSB cultures supplemented with glutamine. Several

suggestions can be offered to explain these results. It would appear that the addition of glutamine inhibits the accumulation of [6]gingerol by affecting its metabolism; it may be also possible that glutamine affects the transport of this compound into the medium and it may be suggested that the formation of conjugate(s) is stimulated which would lower the amount of free PPPs in the medium affecting the quantification of these compounds (see 3.6 and 4.4).

It has been suggested that glutamine may induce organogenesis in some cultures (Lindsey and Jones, 1989) but organogenesis did not occur in these ginger cultures. However, the addition of glutamine may induce biochemical differentiation which changed the pattern of accumulation, so that PPPs were accumulated earlier in the culture cycle when the cells were still in a phase of active growth (see 3.5.3; 3.5.4). This contrasts with the suggestion of Yeoman *et al.* (1980, 1982) that the accumulation of secondary metabolites normally takes place at the cessation of growth and coincides with cell differentiation.

In cultures grown in SH medium without glutamine most of the [6]gingerol is present within the cells and the maximum accumulation occurred at d 30 when cultures were in a phase of active growth. The addition of glutamine delays the accumulation of [6]gingerol in the cells until the stationary phase although some [6]gingerol was present in the medium during active growth and then disappeared. A possible reason for this variation can be explained by a low recovery during extraction possibly due to the presence of conjugate(s) (see 3.6 and 4.4). However, an overall effect of glutamine on metabolism cannot be ruled out and there are numerous reports in the literature which suggest that the composition of the growth medium alters secondary metabolism. For example Sakamura *et al.* (1986) and Sakamura and Suga (1989) have reported changes in the volatile constituents of shoot tip cultures of ginger when incubated on MS and Gamborg's B_5 media supplemented with different combinations of NAA and BAP.

Table 4.3 Effects of the addition of glutamine and medium composition on the maximum accumulation of PPPs (μg flask^{-1})

Medium	PPPs			
	Cell		Medium	
	Time (d)	Amount	Time (d)	Amount
1/2MSB	-	-	46	20
1/2MSBG	12	0.20	24	5
SHB	30	7.3	-	-
SHBG	46	4.2	24	4

4.3.2 Effects of an increase in the level of sucrose

Although the addition of glutamine to different basal salt media supplemented with different combination of PGRs did not stabilise the pH it did alter the pattern of accumulation and localisation of PPPs in cultures.

There are a number of reports in the literature which show that the yield of secondary metabolites can be increased by elevating the sucrose level in the medium. For example Yamakawa *et al.* (1983) reported an increase in the quantity of anthocyanins in *Vitis* suspension cultures when the sucrose concentration was raised. Also Roper *et al.* (1985) showed that nicotine production was increased when the sucrose concentration was elevated from 3% to 4%. If raising the sucrose concentration reduced growth and product yield was linked to growth then it would also decrease product yield.

The expected response of suspension cultures of *Z.officinale* to an increase in the amount of sucrose in the medium was a lower increase in total cell biomass which can be explained as a slower cell division rate resulting in a slower doubling time. However, statistical analysis showed no difference from the control (3% sucrose) and

cultures with 6% and 9% sucrose although there was a difference at the 12% level. This was very likely due to the large variation observed within the control replicates. Moreover, the lower increase in biomass as the sucrose concentration rises (particularly as 12% level) would likely indicate that an osmotic effect is operating which inhibits cell division, or results in a decrease in cell size as reported by Kimbal *et al.* (1975) who showed that cell size decreases as the sugar concentration increased.

The proportion of pigmented cells in the control cultures (3% sucrose) remained almost constant throughout the culture period. This would suggest that the pigmented cells were dividing to keep pace with the total cell population or that non-pigmented cells were differentiating to become pigmented or both. Moreover, raising the concentration of sucrose in the medium to 6% and 9% did not result in an increase in the proportion of pigmented cells. At the 12% level there was a clear decrease in the proportion of pigmented cells due to the inhibitory effect at this high level of sucrose. This was also reflected in the low increase in biomass and the absence of PPPs (see Figs. 3.5.15; 3.5.18) which is consistent with the results of Mantell and Smith (1983) who reported that high sucrose levels inhibited solasodine production in *Solanum aviculare* cultures.

A common problem with plant cell systems is the variability encountered between experiments. In this study PPPs were sometimes recorded only in the medium and not in the cells and also in both cells and medium (3.5.3). Such differences could be related to the transport mechanism involved in the release of these compounds which may in certain circumstances be inactive. There is also evidence (3.6 and 4.4) which suggests the possibility of the conjugation of PPPs which are then released into the medium and these would not be extracted with non-polar solvents. Another sign of variability is that the highest amount of PPPs occurred in actively growing cultures or at the downturn of growth in different experiments.

4.3.3 Effects of the addition of sunflower oil

Several workers have investigated the effects of lipophillic traps on the yield of non-polar compounds. For example Bisson *et al.* (1983) reported an increase in essential oil production in cultures of *Matricaria chamomilla* when a triglyceride phase was added to the cell culture. Berlin *et al.* (1984) have also demonstrated that the addition of miglyol to suspension cultures of *Thuja occidentalis* resulted in the accumulation of some monoterpenoids in the oil phase which are normally released into the medium. In a later paper Berlin and Witte (1988) using the same culture system showed that the use of lipophillic traps (hexadecane and XAD-2) stimulated the accumulation of monoterpenes by *Thuja*.

In this study sunflower oil was added to cultures in an attempt to remove PPPs from the immediate cell environment and tip the balance towards synthesis thereby increasing product yield. As expected it was shown that both [6]gingerol and [6]shogaol accumulated in the oil, a similar result to that obtained by Berlin *et al.* (1984) with *Thuja*. However, with ginger, the addition of sunflower oil did not enhance product yield, in contrast to the results reported by Bisson *et al.* (1983) with *Matricaria chamomilla*. Parr *et al.* (1987) have also reported an overall increase in the extracellular nicotine level in *Nicotiana rustica* cultures when XAD-4 was added. A number of reasons can be offered to explain these results with ginger.

1) The PPPs were simply trapped in the non-polar phase

2) Addition of sunflower oil brought about the release of these compounds by activating transport mechanisms which are non-operative in the control

3) Failure to increase product yield could be because the addition of sunflower oil might prevent cultures from further synthesis of PPPs by altering their metabolism although there was no effect on growth (see Fig. 3.6.4a)

4.4 Fate of [6]gingerol administered to suspension cultures

It has been shown in this thesis that [6]gingerol and related compounds appear and disappear during a culture cycle and that some partitioning exists between cells and medium (3.5.3) when different culture media were used. One possible explanation of these apparently inconsistent results is that [6]gingerol and other PPPs are converted to more polar compounds by conjugation with sugars or other moieties. Such conversions are commonly encountered in plant cell cultures and provide a means whereby the plant can detoxify potentially harmful compounds.

The addition of [6]gingerol at a potentially harmful concentration did not affect the cells visibly, indeed the compound was taken up by the cells and disappeared rapidly. Such an almost complete disappearance of [6]gingerol could be accounted for by its degradation or conjugation with other moieties such as sugars.

However, the possibility exists that some of the [6]gingerol becomes bound to elements in the culture medium preventing extraction. Recovery rates from freshly autoclaved medium and from conditioned medium were 74% and 56% respectively suggesting some binding (see Table 3.6.3). Similar results were reported by Goy (1991) after the addition of codeinone to freshly autoclaved medium and conditioned medium of *Papaver somniferum*. These results suggest that the added [6]gingerol has bound to metabolites present in the conditioned medium possibly forming glycosylates. Furuya *et al.* (1978) have reported the glycosylation of digitoxin by cultures of *Datura purpurea*; and Tabata *et al.* (1988) have also reported the glycosylation of phenolic compounds by plant cell cultures.

The recovery of [6]gingerol, in the presence of cells, was even less efficient than from conditioned medium suggesting that the [6]gingerol has been taken up by the cells and then metabolised. This would be consistent with the results of Kamel *et al.* (1992) who showed a rapid uptake of butyric acid by cultures of *Nicotiana plumbaginifolia* followed by intracellular glycosylation. However the binding of

[6]gingerol and related PPPs to compounds in the medium is still a real possibility and may account for some of the added [6]gingerol. Evidence presented in this study (see 3.6.2.4) showed that none of the known ginger phenolics were detected after acid hydrolysis of the cell residue, showing that any PPPs glycosylated intracellularly were not retained within the cell matrix. Conversely, conjugates of [6]gingerol were present in the medium and these were converted to free derivatives after alkaline or acid hydrolysis. This provides some evidence for the presence of glycosyl derivatives in the cultures which could serve as a method for detoxification of harmful compounds arising from normal plant metabolism (Sandermann *et al.* 1977).

One product of alkaline hydrolysis of the medium of cultures supplied with [6]gingerol was zingerone and this is consistent with the results of Connell and McLachlan (1972) and MacHale (1989) who reported the conversion of [6]gingerol to zingerone by retroaldol condensation. Also, some [6]shogaol was present, although [6]gingerol was not detected after alkaline hydrolysis indicating that it had either been metabolised or totally converted by alkaline hydrolysis. Therefore, it would appear that [6]gingerol was bound via ester bonds to some sugars which is consistent with the results of Sukrasno (1991) who reported the presence of conjugated phenolics present as ester glycosides as well as *O*-glycosides in both chilli cultures and chilli fruits (Sukrasno and Yeoman, 1993).

Similarly, after acid hydrolysis of the liquid medium of cultures supplied with [6]gingerol approximately 14% of PPPs were recovered. [6]shogaol was present which is consistent with the results of Connell and McLachlan (1972) and MacHale (1989) who reported the conversion of [6]gingerol to [6]shogaol via dehydration after acid hydrolysis. This result proved there was a linkage of [6]gingerol to sugars present in the medium via *O*-glycosidic bonds and once again is consistent with the results of Sukrasno (1991) and Sukrasno and Yeoman (1993).

The occurrence of glycosylated forms of PPPs of ginger has not been reported in the literature; however, Wu *et al.* (1990) have reported the occurrence of glycosidically bound aroma compounds of ginger. From the evidence obtained in this study there is no doubt that glycosylated forms of PPPs are present in ginger cultures. These results are similar to those results of Furuya *et al.* (1987) who reported the glycosylation of 2-phenylpropionic acid in a range of suspension cultures; also of Kokubo *et al.* (1991) who reported the glycosylation of quercetin by hybrid cell cultures of *Vitis*. On the other hand, Takahashi *et al.* (1993) have shown the biotransformation of [6]gingerol and [6]shogaol by *Aspergillus niger*. [6]gingerol was converted to the corresponding primary alcohol and [6]shogaol to a ketoalcohol and a diol, but there was no record of glycosylation.

The presence of glycosylated PPPs in cell cultures of ginger could provide some answers to the variations observed in the yield of PPPs in this study, in particular, the partitioning between cells and medium, as well as the absence of PPPs in the late phases of growth where the accumulation of PPPs was initially recorded. Perhaps a higher product yield might have been detected if the separation of conjugated forms, which impaired extraction and therefore detection, had been performed.

4.5 Radioactive feeding experiments

A series of experiments were conducted employing different putative radioactive precursors of the biosynthetic pathway leading to [6]gingerol to compare their effectiveness as precursors and their pattern of incorporation.

4.5.1 Radioactive feeding experiments with ginger rhizome explants employing [U-^{14}C]*p*-coumaric acid

Radioactive feeding experiments employing [U-^{14}C]*p*-coumaric acid failed to label [6]gingerol, in contrast with the results obtained by Denniff *et al.* (1980) who reported the incorporation of [^{14}C] into [6]gingerol employing [U-^{14}C]*p*-coumaric acid. Only one unknown compound was labelled after 24h but this did not correspond with any of the known ginger PPPs. Substantial radioactivity was however, present in the cell residue and this, as expected, increased from 0 to 48h suggesting that the cell wall fraction is a radioactive sink, trapping this precursor, which appeared to be involved in cell wall metabolism. This is consistent with the findings of Sukrasno (1991) who reported a major incorporation of label into lignin-like material and saponifiable cell wall phenolics after feeding [U-^{14}C]*p*-coumaric acid to chilli fruits.

The evidence presented in this study (see Fig. 3.8.1) does show that [^{14}C] was taken up by the cells suggesting the precursor was available for metabolism, provided it was available in the right cell compartment, to ginger PPPs. Indeed TLC analysis showed that a large proportion of the added radioactivity remained at the loading points which could be interpreted as presence of conjugate(s).

Further investigation of the putative conjugate(s) at the loading points could reveal if any conjugated [6]gingerol was present and could explain why the free PPPs were not labelled. Another possibility which might explain the failure of incorporation into free PPPs could be a dilution effect. Any [6]gingerol labelled would be diluted out by the substantial amount of [6]gingerol present in the rhizome blocks and it would be difficult to detect. However, the fact that [6]gingerol and putative zingerone were labelled when [U-^{14}C]cinnamic acid was employed (see 3.8.2; 4.5.3) would make this explanation less likely.

4.5.2 Radioactive feeding experiments with ginger rhizome explants employing [methyl-^{14}C]ferulic acid

Ferulic acid has been described as the most effective precursor of the biosynthetic pathway leading to [6]gingerol (Deniff *et al.*, 1980). This compound is much closer to the end product ([6]gingerol) than *p*-coumaric or cinnamic acid and therefore, according to Lindsey and Yeoman (1984ab) and Yeoman *et al.* (1990), would be expected to be incorporated more effectively. However, as with [U-^{14}C]*p*-coumaric acid [^{14}C-methyl]ferulic acid also failed to label any of the known ginger PPPs.

The distribution of radioactivity was similar to that observed with [U-^{14}C]*p*-coumaric acid with a substantial amount of [^{14}C] remaining a the loading points of the TLC again suggesting the possibility that PPP conjugate(s) are formed confirming the role of this precursor as an intermediate of phenolic conjugates metabolism. However, there is no evidence, in the literature, of the existence of glycosylated ginger phenolics, although evidence in this study with suspension cultures showed the presence of conjugates after the addition of [6]gingerol to cultures. Therefore such conjugates could occur in rhizome tissue but further investigation into this fraction would be necessary to reveal this possibility.

Again as with [U-^{14}C]*p*-coumaric acid there was a substantial amount of radioactivity present in the cell residue showing the role of ferulic acid as an important intermediate in cell wall synthesis as suggested by Lindsey (1986b) who observed a similar fate for [U-^{14}C]cinnamic acid fed to *Capsicum frutescens* cultures, and Fry (1984) in spinach cultured cells. As the precursor has been taken up by the cells, it is available for metabolism although it may not reach the relevant metabolic compartment. Another proposed reason for non-incorporation into the free PPPs is that the labelled methyl group could be removed by a methyltransferase. However, this possibility is not strongly supported by the biosynthetic pathway

proposed by Denniff *et al.* (1980) who showed that the phenolic ring of [6]gingerol derived from ferulic acid.

4.5.3 Radioactive feeding experiments with ginger rhizome explants employing [U-^{14}C]cinnamic acid

Unlike [U-^{14}C]*p*-coumaric acid and [^{14}C-methyl]ferulic acid [U-^{14}C]cinnamic acid, an earlier precursor in the phenylpropanoid pathway than either of these compounds, was incorporated effectively into free [6]gingerol and into a compound tentatively identified as zingerone. This is consistent with the results of Sukrasno (1991) who reported a higher incorporation of [^{14}C] into capsaicin of the chilli pepper fruit when [U-^{14}C]cinnamic acid was used rather than [U-^{14}C]*p*-coumaric acid. Lindsey (1986b) also reported that [U-^{14}C]cinnamic acid was a more effective precursor of capsaicin than [U-^{14}C]phenylalanine when fed to cultures of *C. frutescens*.

The incorporation of [^{14}C] into the soluble cellular fraction was greater with this intermediate than with [U-^{14}C]*p*-coumaric acid. This could have been due to a more effective uptake into the cells and a more favourable transport to the compartment concerned with the biosynthesis of [6]gingerol.

p-Coumaric acid was also labelled which indicates that cinnamate 4-hydroxylase was operative and is consistent with the results of Holland (1989) who reported the activity of this enzyme in cultures of *C.frutescens* accumulating capsaicin. *p*-Coumaric acid was further metabolised as the incorporation of [^{14}C] into [6]gingerol and zingerone suggests. Therefore, it is possible that the addition of [U-^{14}C]cinnamic acid may have activated some of the enzymes of the phenylpropanoid pathway and is consistent with the findings of Lamb (1977) and Gerrish *et al.* (1985) who reported the activation of various enzymes of the phenylpropanoid pathway after the addition of trans-cinnamate to suspension cultures of *Phaseolus vulgaris*.

Similar to the results obtained with [U-^{14}C]*p*-coumaric acid and [^{14}C-methyl]ferulic acid, a substantial amount of [^{14}C] was present in the cell residue (cell wall), indicating another metabolic fate for this precursor, which is consistent with Fry (1984) and Lindsey (1986), who also reported the incorporation of [U-^{14}C]cinnamic acid into the cell wall in spinach and *C.frutescens* suspension cultures respectively. However, some of the ginger PPPs were successfully labelled despite this other metabolic fate.

Once again a substantial amount of radioactivity was present at the loading points on TLC suggesting the presence of conjugate(s). A sharp decrease of [^{14}C] in labelled compounds occurred after 24h which may be explained by further metabolism of the labelled compounds. It was also observed that the decline of [^{14}C] in the cell extract paralleled the increase in the proportion of radioactivity in the cell residue (cell wall) supporting the view that the cell wall is, as expected, acting as a metabolic sink for phenylpropanoids.

4.5.4 Radioactive feeding experiments with suspension cultures employing [U-^{14}C]cinnamic acid

First attempts to label PPPs with [U-^{14}C]cinnamic acid fed to 30d old suspension cultures failed; however, an unknown compound present in the acetone cell extract was labelled.

Similar to the radioactive experiments with rhizome explants a substantial proportion of radioactivity in the cell extract was present at the loading points on the TLC suggesting the possibility of conjugated phenolic(s). Furthermore, a gradual increase in the proportion of [^{14}C] in the polar fraction of the medium supports this possibility.

Here once again the cell residue contained a substantial amount of radioactivity, probably in the cell wall fraction (Fry, 1984 and Lindsey, 1986b).

In the last part of this discussion the results of the radioactive feeding experiments with suspension cultures supplemented with sunflower oil employing [U-^{14}C]cinnamic acid are considered and discussed in relation to those already considered. It has been shown in this study (3.6.3) that the addition of sunflower oil to suspension cultures resulted in the accumulation of PPPs in the lipid fraction. This would lessen the possibility of glycosylation and therefore might result in an increase in labelled PPPs.

4.5.5 Radioactive feeding experiments with suspension cultures supplemented with sunflower oil employing [U-^{14}C]cinnamic acid

In contrast to the results discussed in 4.5.4 here there was incorporation of [^{14}C] into [6]gingerol in 30d old suspension cultures with or without sunflower oil.

The incorporation of [^{14}C] into [6]gingerol suggests, as reported by Holland (1989), that cinnamate 4-hydroxylase was active; however, in contrast to the results obtained with rhizome explants here labelled coumarate was not detected which suggests it is turned over rapidly. It is also possible that the addition of [U-^{14}C]cinnamic acid has activated the enzymes of the pathway resulting in the incorporation of [^{14}C] into [6]gingerol similar to the results obtained with rhizome explants and as suggested by Lamb (1977) and Gerrish *et al.* (1984).

Once again a substantial amount of radioactivity in the cell extract was present at the loading points on TLC suggesting the presence of conjugated phenolic(s). Likewise, the polar fraction of the culture medium also contained considerable amounts of [^{14}C], providing further evidence for glycosylation. Surprisingly there was a significant difference between the amounts of [^{14}C] in the polar fraction of the culture medium of control and treatment from which it would appear that the addition of sunflower oil might have prevented or diminished the occurrence of glycosylation.

The cell residue of cultures with and without oil contained an abundant amount of radioactivity (Fry, 1984 and Lindsey, 1986b). However, despite sunflower oil preventing or diminishing the occurrence of glycosylation and lowering the incorporation of [^{14}C] into the cell residue this did not result in a higher incorporation of [^{14}C] into [6]gingerol. It could be that the considerable amount of [^{14}C] present in the oil extract, which corresponded almost entirely to unincorporated [U-^{14}C]cinnamic acid, reduced the availability of the precursor to the cells and therefore change the amount of [^{14}C] incorporated.

Finally, an explanation can be offered to explain why the first attempt to label PPPs in suspension cultures failed and why the second attempt was successful. The two sets of cultures may have been at different stages of biochemical differentiation which would mean that all the enzymes in the pathway were not fully operative.

4.6 Future work

This study has shown that the cellular localisation of PPPs lies in yellow cells which are mainly distributed in the rhizome of the plant. Established cultures (callus, regenerating and suspension) also showed the presence of this cell type and are likely to be the sites of accumulation of PPPs. Moreover, a decline in the amount of PPPs in suspension cultures was observed. Furthermore, evidence presented in this study was consistent with the formation of conjugated phenolics after the addition of [6]gingerol to suspension cultures. Here, objectives for future work are outlined:

4.6.1 Cytological investigation

The results in this thesis have shown that the number of yellow cells in explants forming callus and regenerating morphological structures appears to decrease with time. However, these estimates were made on 'representative' sections and are subject

to interpretation, clearly an improved method for counting these cells should be devised to study the response of the entire tissue as this would provide sounder data to enable a better understanding of the changes in this cell type during development. Such methods are usually based on the use of reagents to disaggregate the tissue to facilitate cell counting. Chromic acid cannot be used as this destroys/releases the pigment in the cells; thus, a method employing a mixture of enzymes placed in an appropriate osmotic medium would appear to be more favourable as the damage to the coloured cells should be slight.

Specific histochemical methods for [6]gingerol and other PPPs would be devised employing a fluorescent immunohistochemical approach (Drury and Wallington, 1980) exploiting the possibility of conjugating antibodies of PPPs with a fluorescent dye thus producing a fluorescent antibody complex which when applied would combine with the corresponding antigen enabling the precise determination of the site of accumulation of these compounds.

4.6.2 Strategies to enhance secondary product yield

Variation in the accumulation of PPPs between suspension cultures of *Z.officinale* was a common feature in this study therefore a number of strategies should be explored to achieve less variation and to enhance product yield. The following points should be investigated:

a) Suspended cells also exhibited the presence of pigmented cells which were established in rhizome and adventitious roots to be the repository sites of a range of secondary metabolites including PPPs. Assuming this is the case in suspended cells the screening and selection of these cells could enhance product accumulation. Due to the presence of the pigment screening and selection of this cell type would be performed by eye. This would enable an assessment to be made as to whether the synthesis and accumulation of PPPs take place in these cells. Likewise this would

also permit an investigation into whether the synthesis of the yellow colouring matter, suggested to be flavonoid-like compounds, is related to the biosynthesis of PPPs and then it could be established if these compounds share any common intermediate which would influence the balance towards PPPs or the accumulation of flavonoid-like compounds.

b) Cell immobilization of suspension cultures would be carried out to encourage cell differentiation and therefore stimulate product accumulation (Lindsey, 1986a; Lindsey and Yeoman, 1986).

4.6.3 Investigation of the glycosylation of PPPs

Further investigation should be conducted to identify the conjugated phenolics formed in cultures; likewise the possibility of the formation of conjugates in the intact plant should be considered. There is some indication from the radioactive feeding experiments that a substantial amount of radioactivity is present in the cell residue. Further studies would be required to determine whether saponifiable PPPs are present in the cell wall acting as a phenolic 'sink'.

4.6.4 Radioactive feeding experiments

Radioactive feeding experiments with [U-^{14}C]cinnamic acid have shown the effectiveness of this precursor in the biosynthetic pathway leading to [6]gingerol. Time course feeding experiments with this labelled intermediate would be conducted with suspension cultures throughout the growth cycle to assess its metabolic fate and to determine when the biosynthetic pathway is activated. It would also be interesting to study the possible regulatory role of cinnamate 4-hydroxylase during the biosynthesis of [6]gingerol.

CHAPTER 5

APPENDIX

Table 1 Comparison of the changes in PCV of suspended cells cultured in different media and showing different inoculum size. 1/2MSB=control (inoculum 0.5g fw), 1/2MSB* cultures initiated with an inoculum size of 1g, 1/2MSA cultures showing lower amount of 2,4-D (0.5mg l^{-1}) with an inoculum size of 0.5g. Each value is the mean of three replicates ± se after Arcsin transformation

	PCV									
Medium	**0d**	**5d**	**10d**	**15d**	**20d**	**25d**	**30d**	**35d**	**45d**	**50d**
1/2MSB	13.68±0.76^{a}	14.06±0.80^{a}	20.96±0.26^{a}	21.75±0.62^{a}	24.49±0.24^{a}	25.68±0.0^{a}	28.63±0.9^{a}	29.90±0.09^{a}	29.16±0.84^{a}	29.16±0.84^{a}
1/2MSB*	18.19±0.24^{b}	22.22±1.52^{b}	26.27±1.24^{b}	28.14±0.17^{b}	28.73±0.60^{b}	30.23±0.16^{b}	30.70±0.28^{a}	30.33±0.46^{a}	29.93±0.06^{a}	29.59±0.80^{a}
1/2MSA	14.33±0.86^{a}	15.11±0.23^{a}	16.73±0.84^{b}	20.54±1.89^{a}	20.26±0.25^{b}	20.65±1.13^{b}	22.42±0.57^{b}	21.33±1.88^{b}	21.04±1.48^{b}	21.39±0.74^{b}

Values within a column not followed by the same letter differ significantly (P=0.05) by one way analysis of variance (*t*-Test).

Table 2 Comparison of the changes in fw of inoculate cultured on two different regenerating media. Each value is the mean of three replicates ± se

	fw (g)					
Medium	**0d**	**10d**	**20d**	**30d**	**40d**	**50d**
MS 3/0.5	0.024±0.0026^{a}	0.136±0.026^{a}	0.218±0.034^{a}	0.45±0.176^{a}	0.503±0.105^{a}	0.715±0.412^{a}
MS 5/1	0.024±0.0026^{a}	0.179±0.073^{a}	0.310±0.054^{a}	0.324±0.06^{a}	0.160±0.015^{b}	0.173±0.031^{a}

Values within a column not followed by the same letter differ significantly (P=0.05) by one way analysis of variance (*t*-Test)

Table 3 Comparison between the number of yellow pigmented cells in explants from regenerating media. Each value is the mean of three replicates ± se

	No. pigmented cells					
Medium	**0d**	**10d**	**20d**	**30d**	**40d**	**50d**
MS 3/0.5	28.31±3.91[a]	8.41±0.230[a]	6.090±0.160[a]	5.520±2.64[a]	4.190±1.170[a]	2.66±0.070[a]
MS 5/1	28.31±3.91[a]	7.180±4.07[a]	6.980±2.620[a]	6.80±0.140[a]	5.390±0.670[a]	2.10±1.040[a]

Values within a column not followed by the same letter differ significantly (P=0.05) by one way analysis of variance (*t*-Test)

Table 4 Comparison of the changes in fw of inoculate cultured on two different callus induction media. Each value is the mean of three replicates ± se

	fw (g)					
Medium	**0d**	**10d**	**20d**	**30d**	**40d**	**50d**
1/2MSB	0.025±5x10^{-3}[a]	0.08±5.5x10^{-3}[a]	0.099±0.039[a]	0.179±0.27[a]	0.391±0.101[a]	0.9575±0.08[a]
SHB	0.024±1x10^{-3}[a]	0.83±8.5x10^{-3}[a]	0.156±0.012[a]	0.183±0.04[a]	0.507±0.159[a]	1.176±0.098[a]

Values within a column not followed by the same letter differ significantly (P=0.05) by one way analysis of variance (*t*-Test).

Table 5 Comparison of the number of yellow pigmented cells in explants from callus induction cultures. Each value is the mean of three replicates ± se

	No. pigmented cells					
Medium	**0d**	**10d**	**20d**	**30d**	**40d**	**50d**
1/2MSB	18.19±0.61[a]	12.80±4.470[a]	4.050±0.177[a]	7.36±3.00[a]	1.87±1.260[a]	3.61±2.520[a]
SHB	18.19±0.61[a]	17.18±1.120[a]	10.38±6.780[a]	6.39±0.66[a]	0.77±0.660[a]	0.61±0.140[a]

Values within a column not followed by the same letter differ significantly (P=0.05) by one way analysis of variance (*t*-Test).

The data of Tables 3 and 5 where also compared with each other showing statistical difference between 1/2MSB and both MS media on d 30, and MS 3/0.5 on d 30 similarly a comparison between SHB and both MS 3/0.5 and MS 5/1 show differences on d 10.

Table 6 Comparison of the changes in fw (g flask^{-1}) in four different suspension cultures. Each value is the mean of three replicates ± se. 1/2MSB= control

	Time									
Medium	**0d**	**6d**	**12d**	**18d**	**24d**	**30d**	**36d**	**42d**	**48d**	**54d**
1/2MSB	0.763±0.002[a]	0.944±0.006[a]	1.503±0.03[a]	2.22±0.109[a]	2.627±0.20[a]	2.802±0.171[a]	3.553±0.063[a]	3.674±0.047[a]	3.560±0.008[a]	3.40±0.17[a]
1/2MSBG	0.493±0.018[b]	0.770±0.049[b]	1.33±0.041[b]	2.117±0.14[a]	2.958±0.11[a]	3.138±0.081[a]	3.046±0.061[b]	2.380±0.270[b]	3.30±0.059 [b]	3.17.±0.23[a]
SHB	0.440±0.034[b]	0.465±0.037[b]	0.668±0.23[b]	1.209±0.08[b]	2.50±0.295[a]	2.970±0.330[a]	3.550±0.103[a]	3.510±0.286[a]	3.580±0.214[a]	3.30±0.06[a]
SHBG	0.443±0.034[b]	0.62±0.0750[b]	1.47±0.112[a]	2.40±0.222[a]	2.813±0.20[a]	3.510±0.257[a]	3.146±0.467[a]	2.600±0.073[b]	2.240±0.070[b]	2.45±0.26[a]

Values within a column not followed by the same letter differ significantly (P=0.05) by one way analysis of variance (*t*-Test).

Table 7 Comparison of the changes in the amount of [6]gingerol present in four different suspension cultures (μg flask^{-1}). Each value is the mean of three replicates ± se. 1/2MSB= control

Medium	Time									
	0d	6d	12d	18d	24d	30d	36d	42d	48d	54d
1/2MSB	0.087±0.002^{a}	1.294±0.060^{a}	1.217±0.10^{a}	1.206±0.001^{a}	2.385±0.70^{a}	5.155±2.78^{a}	8.070±0.900^{a}	6.180±1.200^{a}	21.64±13.72^{a}	11.40±0.19^{a}
1/2MSBG	0.010±0.010^{b}	0.630±0.330a	3.07±1.540^{a}	5.530±0.520^{a}	0.113±0.06^{b}	0.046±0.02^{b}	0.086±0.003^{b}	0.115±0.061^{b}	0.00±0.00 ^{b}	0.00±0.00 ^{b}
SHB	0.000±0.000^{b}	0.017±0.017^{b}	0.16±0.110^{b}	0.028±0.028^{b}	0.03±0.015^{b}	0.00±0.00 ^{b}	0.360±0.210^{b}	0.106±0.106^{b}	0.00±0.00 ^{b}	0.00±0.00 ^{b}
SHBG	0.000±0.000^{b}	0.000±0.0 0 ^{b}	1.71±1.260^{a}	0.440±0.310^{a}	4.15±1.33^{b*}	0.89±0.750^{b}	0.00±0.00 ^{b}	0.055±0.055^{b}	0.183±0.11 ^{b}	0.00±0.00 ^{b}

Values within a column not followed by the same letter differ significantly (P=0.05) by one way analysis of variance (*t*-Test), or at P=0.1 (*).

Table 8 Comparison of the changes in fw (g flask^{-1}) of suspension cultures grown in media with increasing amount of sucrose. Each value is the mean of three replicates ± se. 1/2MSB= control (3% sucrose)

Medium	Time								
	0d	6d	12d	18d	24d	30d	36d	42d	48d
1/2MSB	0.41o±0.057^{a}	0.513±0.064^{a}	1.506±0.224^{a}	1.983±0.243^{a}	3.757±1.140^{a}	4.040±1.690^{a}	4.21±1.1470^{a}	4.196±1.080^{a}	4.226±1.31^{a}
1/2MSB6%	0.341±0.068$^{a\Phi}$	0.61±0.122 $^{a\Phi}$	1.195±0.281$^{a\Phi}$	1.470±0.014$^{a\Phi}$	1.840±0.157$^{a\Phi}$	2.650±0.276$^{a\Phi}$	2.272±0.094$^{a\Phi}$	2.77±0.2670$^{a\Phi}$	3.429±0.41$^{a\Phi}$
1/2MSB9%	0.345±0.068$^{a\Phi}$	0.514±0.039$^{a\Phi}$	1.013±0.093$^{b\Phi}$	1.266±0.257$^{b\Phi}$	1.269±0.195$^{a\Phi}$	1.730±0.190$^{a\diamond}$	2.259±0.279$^{b*\Phi}$	2.214±0.056$^{b*\Phi}$	2.535±0.22$^{a\Phi}$
1/2MSB12%	0.552±0.028$^{a\Phi}$	0.604±0.129$^{a\Phi}$	0.730±0.131$^{b*\Phi}$	0.710±0.100$^{b\diamond}$	1.283±0.108$^{b*\diamond}$	1.381±0.316$^{a\diamond}$	1.620±0.230$^{b*\diamond}$	1.172±0.163$^{b*\diamond}$	2.54±0.220$^{a\Phi}$

Values within a column not followed by the same letter differ significantly from the control (at P=0.05) by one way analysis of variance (*t*-Test), or at P=0.1 (*). Treatments within a column with the same symbol do not differ significantly (at P=0.05) from each other.

Table 9 Comparison of the changes in PCV of suspension cultures grown in media with increasing amount of sucrose. Each value is the mean of three replicates ± se after Arcsin transformation

Medium	Time								
	0d	6d	12d	18d	24d	30d	36d	42d	48d
1/2MSB	12.83±0.08^{a}	13.17±0.25^{a}	18.27±0.16^{a}	19.48±0.47^{a}	27.35±3.73^{a}	27.77±4.99^{a}	28.49±4.69^{a}	29.04±3.97^{a}	29.62±4.06^{a}
1/2MSB6%	13.07±0.15^{a}	13.76±0.42^{a}	14.66±0.34^{b}	20.01±0.44^{a}	20.47±0.45^{a}	24.91±0.43^{a}	23.90±0.57^{a}	23.80±0.94^{a}	27.99±1.26^{a}
1/2MSB9%	12.98±0.06^{a}	13.20±0.16^{a}	13.84±0.36^{b}	15.96±1.32^{b}	17.81±1.07^{a}	19.75±0.77^{a}	21.75±0.60^{a}	20.70±0.24^{a}	22.87±1.17^{a}
1/2MSB12%	11.37±0.44^{b}	12.53±1.29^{a}	13.60±0.22^{b}	15.33±0.32^{b}	16.20±0.48^{b}	16.97±0.89^{b}	17.70±0.71^{b*}	18.40±0.14^{b*}	20.70±0.24^{b*}

Values within a column not followed by the same letter differ significantly from the control (at P=0.05) by one way analysis of variance (*t*-Test), or at P=0.1 (*).

Table 10 Comparison of the changes in the number of pigmented cells of suspension cultures grown in media with increasing amount of sucrose. Each value is the mean of three replicates ± se. 1/2MSB= control (3% sucrose)

Medium	Time								
	0d	6d	12d	18d	24d	30d	36d	42d	48d
1/2MSB	15.47±1.482^{a}	18.7±0.680^{a}	15.38±1.972^{a}	18.51±0.614^{a}	17.93±2.10^{a}	17.17±2.03^{a}	17.18±2.46^{a}	15.84±2.09^{a}	14.796±1.74^{a}
1/2MSB6%	15.72±0.515$^{a✞}$	13.69±1.49$^{b✞}$	17.07±1.362$^{a✞}$	17.10±2.290$^{a✞}$	30.49±0.64$^{a✞}$	19.02±4.73$^{a✞}$	23.42±0.21$^{a✞}$	22.29±1.27$^{a✞}$	17.12±3.250$^{a✞}$
1/2MSB9%	15.70±0.510$^{a✞}$	15.89±1.53$^{a✞}$	16.85±1.040$^{a✞}$	17.61±2.930$^{a✞}$	32.63±0.87$^{a✞}$	18.82±4.26$^{a✞}$	25.50±2.48$^{a✞}$	19.50±1.44$^{a✞}$	20.09±1.050$^{a✞}$
1/2MSB12%	17.44±0.570$^{a✞}$	19.72±3.27$^{a✞}$	16.79±0.650$^{a✞}$	17.37±0.520$^{b✞}$	11.76±0.89$^{b*✧}$	10.64±1.22$^{b✧}$	11.77±0.82$^{a✧}$	9.80±0.800$^{b✧}$	9.740±0.149$^{b✧}$

Values within a column not followed by the same letter differ significantly from the control (at P=0.05) by one way analysis of variance (*t*-Test), or at P=0.1 (*). Treaments within a column with the same symbol do not differ significantly (at P=0.05) from each other.

CHAPTER 6

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CHAPTER 7

PUBLICATIONS

Details of publications:

1.- Rafael Zarate, Sukrasno and Michael M. Yeoman (1991). Application of two rapid techniques of column chromatography to separate the pungent principles of ginger *Zingiber officinale* Roscoe, J. Chrom., 609, pp. 407-413.

2.- Rafael Zarate and Michael M. Yeoman (1994). Studies of the cellular localization of the phenolic pungent principle of ginger, *Zingiber officinale* Roscoe, New Phytol., 126, pp. 295-300.

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Short Communication

Application of two rapid techniques of column chromatography to separate the pungent principles of ginger, *Zingiber officinale* Roscoe

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ABSTRACT

Two rapid techniques of column chromatography, flash chromatography and vacuum chromatography, together with solvent systems differing in polarity, have been used to separate the pungent principles (gingerols and shogaols) of an extract of ginger powder. Gingerols consist of a homologous series of aldols each containing a phenolic group which together with shogaols, showing similar structure, give the pungency of ginger. Thin-layer chromatographic analysis of the fractions collected from both chromatographic techniques showed that the main gingerol homologues are separated satisfactorily from the other compounds only using vacuum chromatography. These compounds were eluted with hexane–diethyl ether (30:70) and (20:80) respectively. The results obtained with flash chromatography, on the other hand, were not satisfactory.

INTRODUCTION

The rapid and successful separation of selected compounds from a plant extract containing a myriad of substances is a problem facing the plant biochemist. It is therefore important to effect a crude separation of the plant components before more sophisticated procedures are employed. Such separation may include extraction using selected solvents, partition into two immiscible solvents (liquid–liquid extraction) and vacuum or flash chromatography [1–4]. Apart from the speed of the chromatographic process, vacuum and flash chromatography also offer a high loading capacity. Their use is of course not limited to the crude separation of the components of plant extracts, as they have been used successfully for the purification and separation of compounds in mixtures containing few components, such as products of chemical synthesis. A wide range of packing materials have been employed with both techniques, with silica gel used most frequently, but others, such as cellulose, polyamide, and bonded silica (reversed-phase particles) have also been used [5].

Attempts have been made to isolate gingerols (Fig. 1) from a crude extract of ginger by Shoji *et al.* [6] and Farthing and O'Neill [7]. Shoji *et al.* [6] partitioned the methanol extract between ethyl acetate–water, and *n*-hexane–methanol, followed by repetitive column chromatography (six times) on

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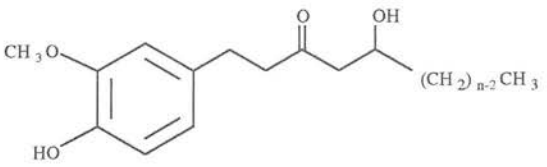
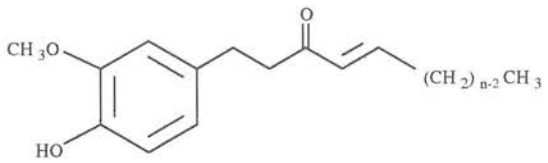


[n]-Gingerol



[n]-Shogaol

Fig. 1. Molecular structure of gingerol and shogaol.

silica gel eluted with benzene–ethyl acetate, while Farthin and O'Neill [7] used the same liquid–liquid extraction [6], followed by counter current chromatography, which gave good separation of the gingerol homologues.

In this paper a comparison is made between flash and vacuum column chromatography and their effectiveness assessed in separating the gingerols the main components of the pungent principle of *Zingiber officinale* Roscoe.

EXPERIMENTAL

Preparation of extract (stock solution)

Freeze-dried ginger rhizome powder (20 g) was extracted with acetone (analytical-reagent grade; BDH, Poole, U.K.) according to the method described by Spiro and Kandiah [8]. The acetone extract was evaporated to dryness on a water bath at 35°C using a rotary evaporator and the residue dissolved in 50 ml of 80% methanol. The resultant solution was subjected to liquid–liquid extraction 3 times with 50 ml hexane. The hexane fraction which contains mainly waxy materials, volatile oils and higher amount of shogaols than in the ether phase, was analysed on thin-layer chromatography (TLC) (see Fig. 3) and then discarded. To the 80% methanol fraction, a mixture of diethyl ether and water (2:1, v/v) was added to produce two layers, the organic and aqueous phases. The pungent principles were mainly present in the ether fraction which was then dried with anhydrous sodium sulphate, evaporated to dryness as before, dissolved in 10 ml metanol and stored as the stock solution.

Thin-layer chromatography

TLC was used to evaluate the products of fractionation and eluates of flash and vacuum column chromatography. TLC was carried out on Silica gel 60, 20 × 20 cm plates, layer thickness 0.2 mm (Merck No. 5553, Darmstadt, Germany), under saturated conditions in a glass tank (Panglas, Shandon, TLC chromatank, Runcorn, UK). The solvent system was toluene–methanol (80:5), a variation of that of Bhagya [9]. Visualization of the compounds on the plates was achieved after spraying with Folin–Ciocalteu reagent (BDH). Using this method gingerols, which constitute the main part of the pungent principle in ginger, displayed R_F values of 0.24–0.29 and shogaols, the minor component, showed R_F values of 0.45–0.50 as shown in Fig. 3.

Flash chromatography

The equipment used for flash chromatography was a glass chromatography column 135 cm in length with an inside diameter of 2.0 cm, supplied by Aldrich (Gillingham, UK), as decribed by Still *et al.* [3]. To prepare the column 9.5 g of silica gel 60 (40–60 μm, Merck 9385) was added slowly while tapping continuously to produce even packing, to give and effective column length of 15 cm, then an 0.8-cm layer of 40–100 mesh sand (BDH) was carefully placed on the flat top of the dry gel bed. Initially, 40 ml of the solvent system, as used for TLC, was added to the column and pressure applied from a cylinder containing nitrogen to remove the solvent from the silica, remove any trapped air and produce a compact column. Finally, an extra 200 ml of solvent was run through, to achieve an even compact column. To the column a 2-ml sample, obtained by evaporating 2 ml of stock solution in methanol in a stream of nitrogen and redissolving the residue in 2 ml of eluent, was applied. The sample was drawn into the silica by applying pressure from a nitrogen

cylinder. After the whole sample had been adsorbed by the column, 200 ml of eluent was added, pressure applied to produce a flow-rate of 50 mm per min (read from the decrease in the level of the solvent above the column bed) and 20 fractions of a volume of 10 ml collected. The compounds in each fraction were analysed by TLC following the method described above.

Vacuum chromatography

A Buchner flask (100 ml) fitted with a sintered filter funnel with an inside diameter of 3.5 cm was connected to a water pump. To the funnel, a similar amount (9.5 g) of silica gel 60, as used in flash chromatography, was added slowly to obtain an evenly packed silica column, 3 cm in length.

A 3-ml volume of the stock solution of ginger extract was added to 2 g of silica gel 60 and dried by means of a rotary evaporator over a warm water bath at 35°C. The dried powder containing the sample was then spread evenly over the top of the silica column. A piece of filter paper (Whatman No. 1, Maidstone, UK) with the same diameter as the inside diameter of the funnel was placed on top of the sample to prevent damage to the column during addition of the solvent. Hexane (25 ml) was added slowly, and the solvent allowed to penetrate the whole column, then the atmosphere in the flask was sucked out to facilitate rapid elution and the eluent collected as the first fraction. Further elution was carried out using 20 aliquots of 25 ml of solvent mixtures with increasing polarity, composed of hexane–diethyl ether and diethyl ether–methanol, as shown in Fig. 2. The individual eluates of 25 ml were transferred to a test tube, evaporated to a volume of 10 ml in a stream of nitrogen and then analysed by TLC.

RESULTS AND DISCUSSION

Preliminary separation

The TLC chromatograms of the fractionation

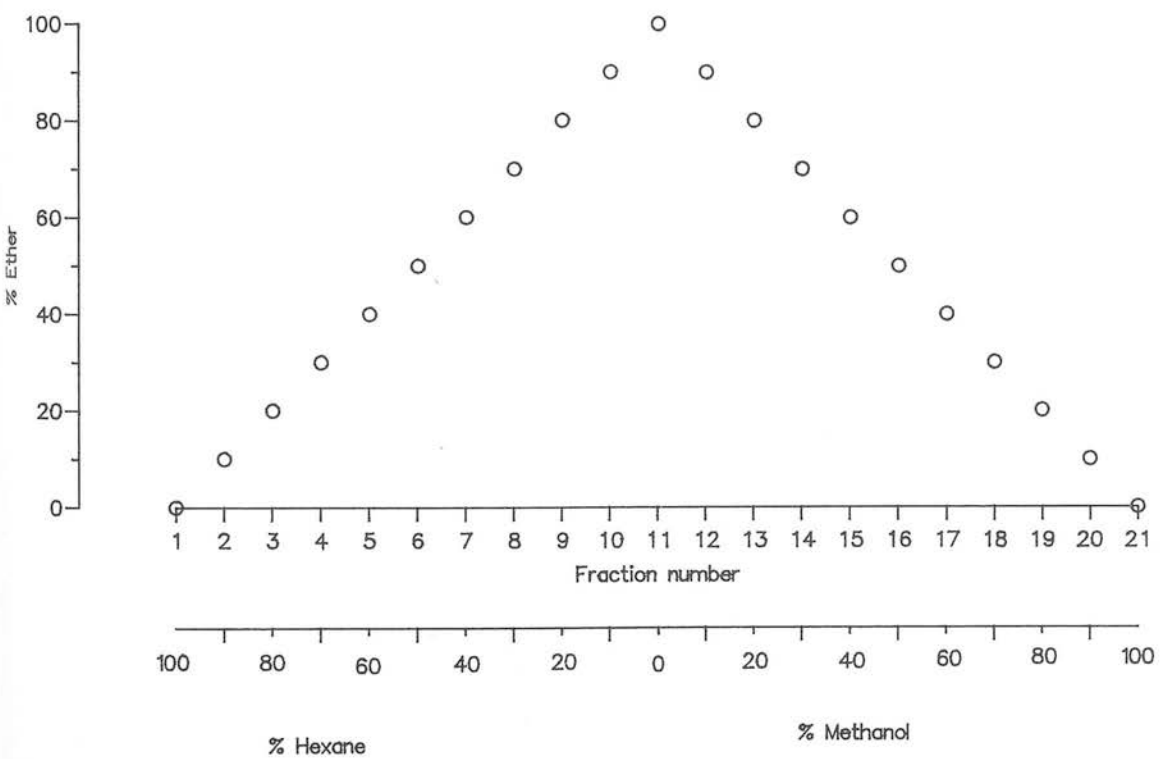


Fig. 2. Composition of the solvent system used in vacuum chromatography (21 different aliquots).

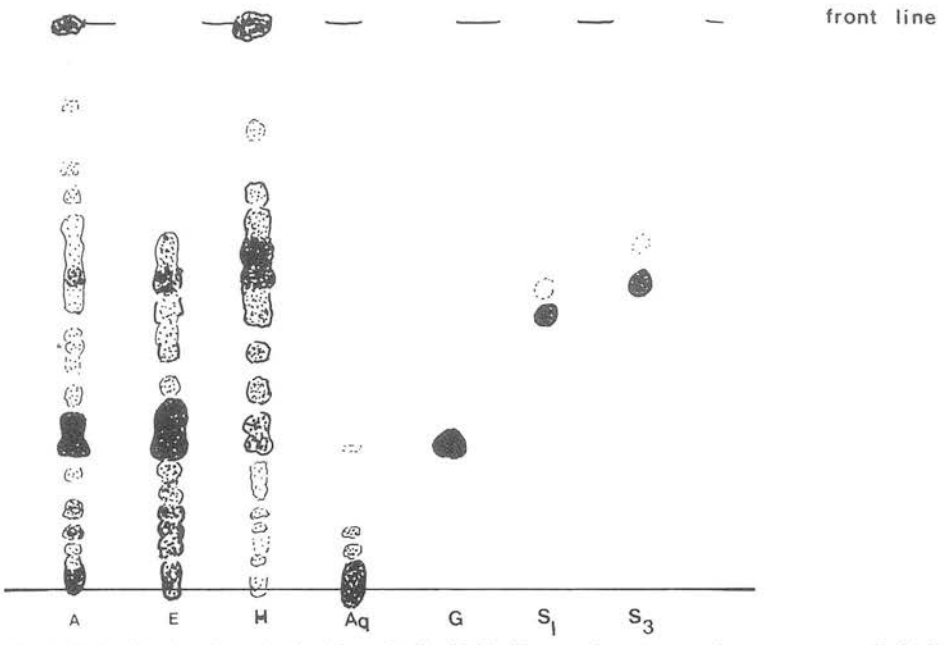


Fig. 3. TLC of the fractions obtained from the liquid–liquid extraction. A = total acetone extract (5 μl), B = ether phase (10 μl), H = hexane phase (10 μl), Aq = aqueous phase (10 μl), G = [6]-gingerol (2 μl = 70 μg), S_1,S_3 = shogaol homologues (2 μl = 25 μg). The amount of sample loaded is indicated in brackets, as for Figs. 4–6.

products are presented in Fig. 3. As predicted, the pungent principles are present mainly in the ether fraction, with a higher amount of shogaol in the hexane phase. In the aqueous fraction, compounds which react with Folin–Ciocalteu reagent were also present. These compounds, however, are unlikely to be the pungent components of ginger, since they do not run far from the origin in the TLC system used.

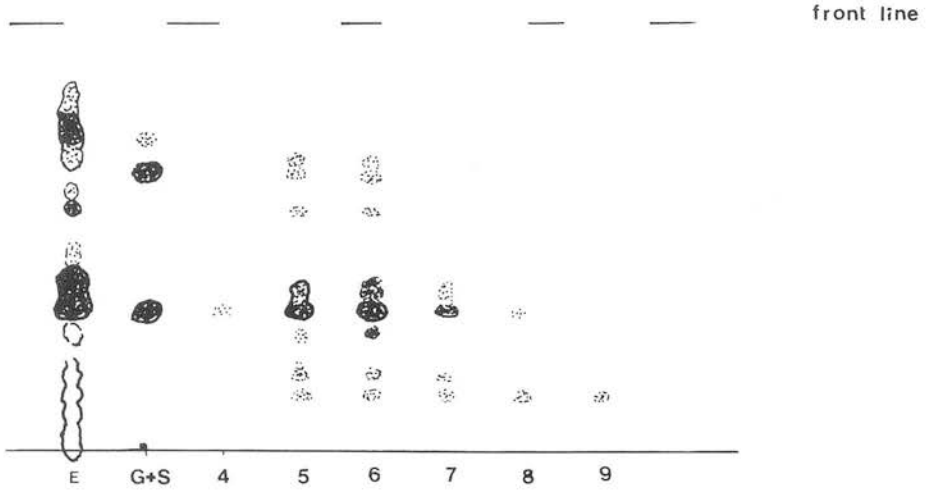


Fig. 4. TLC of the 10-ml fractions obtained using flash chromatography with toluene–methanol (80:5) as solvent. Numbers indicate the different fractions collected (10 μl), E = ether phase (10 μl), G + S = [6]-gingerol (2 μl = 70 μg), shogaol (2 μl = 25 μg). Only fractions which were known to contain the compounds under investigation were loaded onto the TLC plates.

Flash chromatography

This is a moderate-resolution, preparative technique usually carried out in a column-overload condition, using the same solvent system as with TLC. It has been reported that compounds wich differ in R_F value by only 0.15 can be separated satisfactorily using flash chromatography [3,5]. Therefore, it was expected that, using this technique, gingerols the major components of the pungent principle in ginger and shogaols, would be clearly separated because the difference between their R_F values is greater than 0.15 (*ca.* 0.20–0.25).

The results, however, presented in Fig. 4 show that most of the components of the pungent principle (gingerols, shogaols) were present in the same fractions (5 to 7). Overloading does not seem to be the cause, because the loading capacity of the column used [3] is approximately 400 mg, while the amount of sample loaded was less than 200 mg. Different lengths of the bed column (10–15 cm) and different flow-rates (50–100 mm per min) were tested, as well as the solvent system hexane–diethyl ether (30:70) employed in vacuum chromatography which gave clear separation of the gingerols. However, less satisfactory results were obtained as all the components of the pungent principle appear in fractions H_2–H_4 (see Fig. 6). The silica gel employed for the development of the TLC was 15 μm (Merck No. 5553), while for flash chromatography it was 40–60 μm. (Merck No. 9385). If the silica gel used for both forms of chromatography had been the same, as suggested by Majors and Enzweiler [5], it would have simplified comparison of the results. It is also possible that the number of compounds present in the plant extract may also affect the efficiency of the column.

Vacuum chromatography

It was surprising that although the technique of vacuum chromatography is much simpler than flash chromatography, the main pungent components of ginger, the gingerols, are almost completely resolved from the shogaols, present in fractions 8 and 9 (see Fig. 5). Using this technique the length of the column was only 3 cm, much shorter than the column used for flash chromatography (15 cm), with a wider inside diameter of the column which provides a high loading capacity. It appears that the solvent

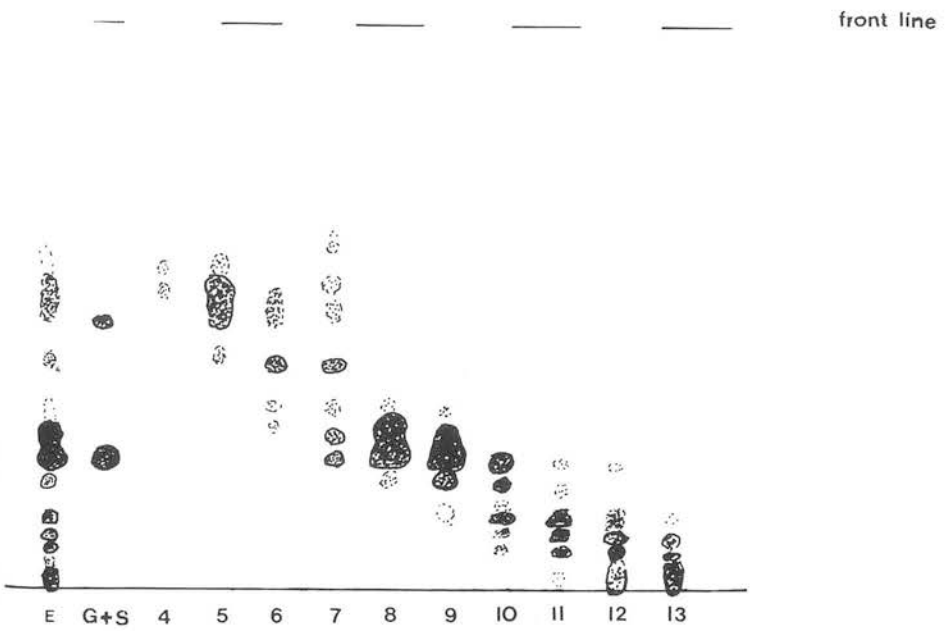


Fig. 5. TLC of the 10-ml fractions obtained using vacuum chromatography, when a solvent gradient was employed. Numbers indicate the different fractions collected, 4–13 (10 μl); E = ether phase (10 μl); G + S = [6]-gingerol (2 μl = 70 μg); shogaol (2 μl = 25 μg). Only fractions which were known to contain the compounds under investigation were loaded onto the TLC-plates.

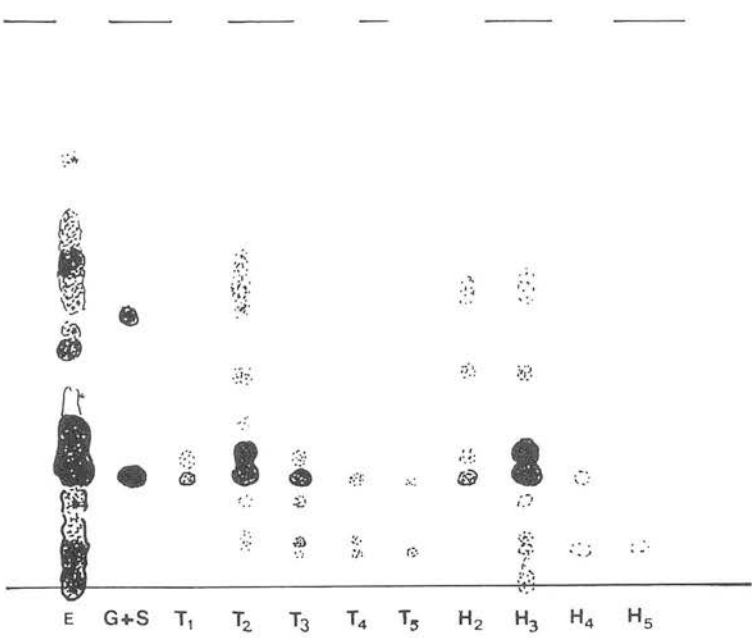


Fig. 6. TLC of the 10 ml fractions obtained using flash chromatography with hexane–diethyl ether (30:70) as solvent system, fractions H_2–H_4 (10 μl) and the 10-ml fractions collected in vacuum chromatography when an isocratic solvent system toluene–methanol (80:5) was used, fractions T_1–T_5 (10 μl); E = ether phase (10 μ); G + S = [6]-gingerol (2 μl = 70 μg); shogaol (2 μl = 25 μg). Only fractions which were known to contain the compounds under investigation were loaded onto the TLC plates.

gradient plays a major part in this chromatographic technique. Indeed, isocratic elution using the solvent system developed for TLC has also been tried. However, a satisfactory separation was not achieved (see Fig. 6). Similar results were obtained in an attempt to isolate the steroid (23,24-dimethylcholesta-5,22-dien-3β-ol) from a petroleum ether extract of *Cresentia cujete* bark (unpublished observation). It is unlikely that the method of application of the sample to the column has any significant effect on the efficiency of separation because the results presented in Fig. 6 (fractions T_1–T_5) show that the separation achieved using an isocratic system was inferior to that with a gradient (see Fig. 5) with the same loading arrangement used for both solvent systems.

Of course, solvent gradients can also be employed in flash chromatography, but then the technique becomes long and tedious, with the simple equipment employed for the above experiments. These results suggest that a number of advantages are offered by vacuum column chromatography. 1) The equipment is of low cost and easily assembled using components available in most laboratories. 2) A solvent system can easily be developed by mixing two solvents with different polarities in different proportions. 3) This method may also be used for purification by increasing the polarity of the gradient more slowly and eluting smaller volumes for each fraction.

A much more complicated system of vacuum liquid chromatography has been developed by Targett *et al.* [4] using a longer column, maintained under vacuum, with isocratic elution as for flash chromatography. Therefore, results similar to those of flash chromatography might be expected since isocratic elution is used, but would appear to be less favourable for a preliminary separation of the component of plant extracts.

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Studies of the cellular localization of the phenolic pungent principle of ginger, *Zingiber officinale* Roscoe

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SUMMARY

Examination of cryo-sections from mature rhizomes, immature rhizomes and adventitious roots of ginger, *Zingiber officinale* Roscoe, showed the presence of yellow-pigmented cells. There is a correlation between the number of pigmented cells and the amount of [6]-gingerol, the main pungent principle in ginger, in these organs. It is also shown histochemically and microspectrophotometrically that the yellow cells contain, in addition to flavonoid-like compounds, possibly curcumin derivatives, phenolics and large amounts of lipid material. Low-temperature scanning electron microscopy also demonstrates the oil content of these cells. Therefore, it would appear that the site of accumulation of flavonoids, curcumin derivatives, phenolics including gingerol and the essential oils of this spice lies within the same cell type.

Key words: *Zingiber officinale*, gingerol, oil cells, yellow cells, accumulation site.

INTRODUCTION

Ginger, *Zingiber officinale* Roscoe, is a monocotyledonous herbaceous plant belonging to the family Zingiberaceae. It is an important commercial species and has been used as a source of spice and medicine in China and India since ancient times (Purseglove *et al.*, 1981). The rhizome contains both the flavour and pungency of the spice together with the essential oils. Steam distillation of rhizome tissue removes the essential oils which represent 1·5–2·5 % of the dry weight; whereas the oleoresin, which is the material extracted with organic solvents, contains chiefly the pungent compounds representing 4·5–7·5 % of the dry weight (Govindarajan, 1982). Gingerols and shogaols give ginger its pungency and consist of a homologous series of aldols each containing a phenolic group. It has been suggested that these compounds are stored in cells which are distributed within the rhizome in both the cortex and pith among a group of hyaline cells (Mangalakumari, Ninan & Mathew, 1984) characterized by their yellow contents and reaction to various reagents; however, these results are inconclusive.

In this paper an attempt has been made to determine the cellular localization of the pungent principle using reagents which are known to react with aldols and by correlating the amount of gingerol in various cell populations with the number of pigmented cells within the population giving a positive response to these reagents. The distribution of the cells containing the essential oils has also been studied using histochemistry together with low-temperature scanning electron microscopy. We report for the first time the accumulation of a series of secondary compounds of ginger within lipid bodies.

MATERIALS AND METHODS

Plant material

The plant material used in this study was removed from mature rhizomes of *Zingiber officinale* Roscoe, 3–4 months old, cultivated in a greenhouse; suspension cultures of ginger; young rhizomes from micropropagated ginger plants and adventitious roots from the mature ginger rhizome.

Petals of flowers of four different species: *Rosa canina* L.; *Senecio cineraria* DC.; *S. jacobaea* L. and *Verbascum blattaria* L. were used for the extraction of flavonoids for spectrophotometric studies.

Extraction of [6]-gingerol

After observation under the microscope cryo-sections of the rhizome were removed from the microscope slide using a scalpel and placed, together with the remaining unsectioned material, in 40–45 ml acetone (Analytical grade, Fisons, Lough-

borough, UK) in a 100 ml Erlenmeyer flask sealed with aluminium foil and stirred for 3 h at room temperature *c.* 22 °C. The tissue was then removed by filtration through a Whatman no. 1 filter paper (Whatman, Kent, UK), and the extract evaporated to dryness using a vacuum rotary evaporator over a warm water bath at 35 °C. Finally, the sample was taken up in 1 ml of methanol (HPLC grade, BDH, Poole, UK) and stored at −20 °C until required for further analysis.

Separation and quantitative analysis of [6]-gingerol

The amount of [6]-gingerol, the main pungent principle of ginger, was determined by high-performance liquid chromatography using a Gilson 302 liquid chromatograph fitted with a C18 lichrosorb reverse-phase column 200 × 4·6 mm i.d. (Capital HPLC, Bathgate, Scotland, UK) and a Gilson 111B u.v. detector. The system was controlled by an IBM PS2 computer. All data processing was performed using the same software. Elution of the sample was carried out using a solvent gradient methanol-water from 65 to 100 %, a variation of the method by Chen, Robert & Chi-Tang (1986) and detection made at 282 nm.

Preparation of cryo-sections and light microscopy

Cylindrical pieces of ginger rhizome from the cortex and medulla, from micropropagated rhizomes and from roots, approximately 4 × 5 mm and 45–50 mg in fresh weight, were taken, mounted onto a specimen stub 12·5 mm in diameter (Agar Scientific Ltd, Essex, UK) with a cryo-adhesive (Tissue-Tek Agar Scientific Ltd) and immediately frozen by immersion in liquid nitrogen for 3–4 min. The stubs were then placed in a cryostat set up at −17 °C. After equilibration of the temperature, 30 sections of 25–30 μm in thickness were sectioned from the top, middle and lower parts of the tissue cylinder (10 sections from each area) and mounted on a microscope slide in a drop of water. Once the sections had thawed they were ready for observation using a Vickers monocular compound microscope (Vickers Instruments Ltd, London) at a magnification of × 200. The number of yellow pigmented cells present in the 30 cryo-sections, from mature rhizome, micropropagated rhizome and mature roots, was counted.

Microspectrophotometric measurements

Microspectrophotometric analysis of the cell contents of individual yellow cells of the cryo-sectioned plant material was performed using a Vickers M85 scanning integrated microdensitometer (Vickers Instruments Ltd, London). Preparation of the sections for these measurements was as described above. An absorption spectrum was obtained for individual cells by measuring the integrated optical density (IOD) over a wavelength range of 400–700 nm at 10 nm intervals.

Histochemical analysis

Several histochemical reagents were employed in attempts to identify the range of compounds present within the cells. It has been reported (Mangalakumari *et al.*, 1984) that a 10 % (w/v) solution of sodium carbonate when applied to tissue sections produces a change in colour of the content of some ginger cells from yellow to red. However, this is likely to be due to the presence of flavonoid-like compounds and not gingerol or shogaol. In order to detect phenolic compounds a mixture of 1 % (w/v) ferric chloride and 0·5 % (w/v) potassium ferricyanide in water was employed. Phenolics give a blue colour with this reagent. Finally, for the localization of lipidic material Nile red (Greenspan, Mayer & Fowler, 1985) (Sigma Chemical Co. Ltd, Poole, UK) was used. A 1 mg ml^{-1} solution of Nile red in 10 ml of acetone (Analytical grade, Fisons, UK) was prepared and from this concentrated solution, 10 μl was taken and dissolved in 2 ml of water; this final solution was used for staining the sections which were examined by fluorescence microscopy using green light excitation (Olympus 1M, G filter), which produces a red fluorescence with lipids.

Cell culture

Suspended cells of ginger were cultured in liquid medium composed of half-strength Murashige & Skoog (1962) basal medium with 3 % sucrose (BDH, UK) supplemented with 1 mg l^{-1} dichlorophenoxyacetic acid (2,4-D) and 0·5 mg l^{-1} benzylaminopurine (BAP), the pH of the medium was adjusted to 5·8 with 1 N KOH or 1 N HCl before autoclaving. Cultures were grown in 40 ml of medium in 250 ml Erlenmeyer flasks sealed with a double layer of aluminium foil and placed on a shaker at 98 r.p.m. in a culture room under the following conditions: temperature 25 ± 2 °C, light intensity 25 μmol m^{-2} sec^{-1} photon flux density (Compton Warmwhite fluorescent tubes).

Spectrophotometry

The absorbance of the samples was measured using a SP8-100 PYE UNICAM ultraviolet spectrophotometer within a wavelength range of 200–600 nm.

Low-temperature scanning electron microscopy

Low-temperature scanning electron microscopy (LTSEM) was performed using a Cambridge Stereoscan S250 microscope (Cambridge Scientific Instru-

ments Co., Cambridge, UK) and an EMscope cryo-preparation system SP2000 (Biorad Microscience Division, UK).

Hand sections from a mature ginger rhizome 1–2 mm in thickness were mounted with cryo-adhesive (Tissue-Tek) on stubs with a series of surface grooves (Jeffree & Read, 1991). The specimen was then frozen by immersion in slushy pre-cooled nitrogen under a dry argon atmosphere at −210 °C for 5–7 min, followed by fracture in a preparation chamber under vacuum. The frozen material was then etched by sublimation of the water under controlled conditions at −70 °C for 10–15 min. Finally, the etched specimens were gold coated and examined under LTSEM.

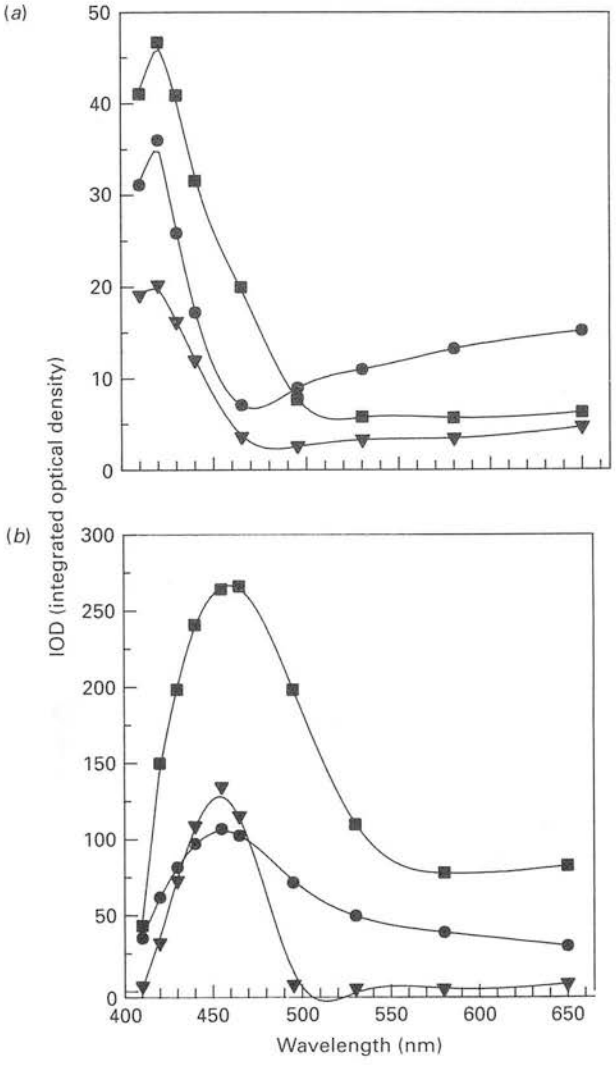

Figure 1. Spectrophotometric measurements of three individual yellow-pigmented cells in a cryo-section: (**a**) before the addition of 10% sodium carbonate solution showing a maximum at 420 nm; (**b**) after the addition of 10% sodium carbonate solution which produces a shift toward the red with a maximum at 455 nm.

Extraction of flavonoids

Samples of flower petals (13 g) of four different species; *Rosa canina*; *Senecio cineraria*; *S. jacobaea* and *Verbascum blattaria*, were employed for the extraction of flavonoids. The petals were homogenized in 100 ml methanol (Analytical grade, BDH) using an electric blender at maximum speed 4× for 1 min at 20 s intervals. The homogenate was then stirred overnight in 500 ml methanol at room temperature. The combined extract was filtered through a Whatman no. 1 filter paper and evaporated to dryness using a vacuum rotary evaporator over a warm water bath at 35 °C. The residue was then dissolved in 100 ml of water and partitioned 3× with equal volumes of chloroform (Analytical grade, BDH). The aqueous fraction was again partitioned 3× with equal volumes of ethyl acetate (Analytical grade, BDH). The final three fractions were evaporated to dryness as described above and taken up in 3 ml of water; the remaining two fractions (chloroform, ethylacetate) were taken up in 3 ml 80% (v/v) methanol–water and stored in the freezer at −20 °C.

RESULTS AND DISCUSSION

It has been reported by Mangalakumari *et al.* (1984) that the colour of the yellow cells becomes brighter and shifts toward the red after the addition of a 10% (w/v) solution of sodium carbonate (see Fig. 3*d*). In this study cells were counted after the addition of sodium carbonate to the sections as the intensifi-

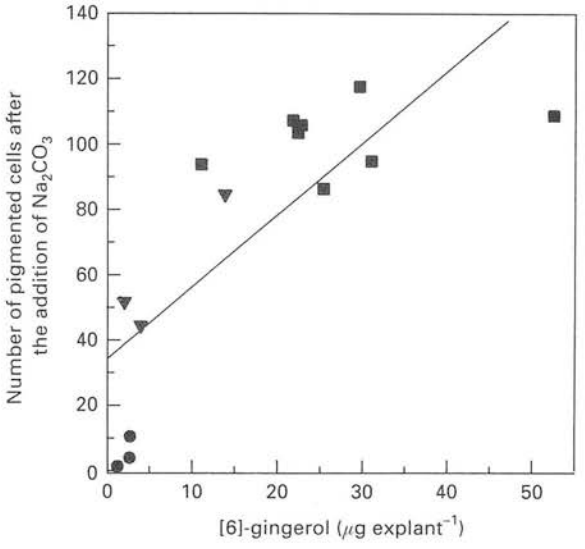

Figure 2. Graph showing the number of pigmented cells after the addition of sodium carbonate in the 30 cryo-sections examined and the amount of [6]-gingerol present within the sections and the remaining unsectioned tissue; the regression line ($y = 2{\cdot}2x + 34{\cdot}3$) shows a correlation with a regression coefficient of 0·7823. (■) cryo-sections from mature rhizomes, (●) cryo-sections from adventitious mature roots, (▼) cryo-sections from micro-propagated rhizomes.

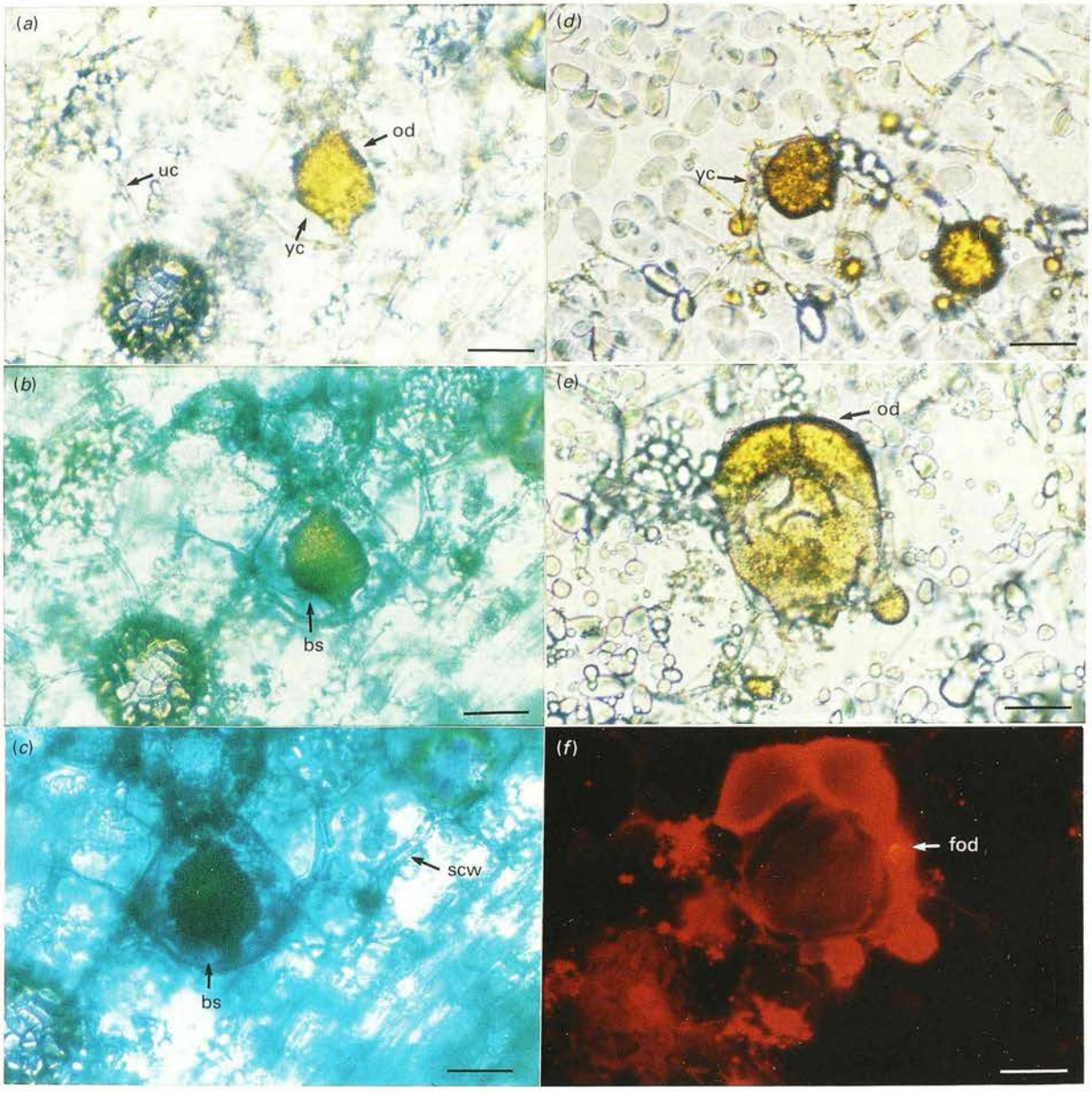

Figure 3. Cryo-sections from a ginger rhizome. (**a**) yellow pigmented cell; observe the yellow colour and its absence in the other cells. (**b**) the same cell as 3**a** photographed 30 s after the addition of a mixture of 1 % ferric chloride and 0·5 % potassium ferricyanide. A blue colour, indicating the presence of phenolic compounds, begins to develop within the yellow compartment as well as in the walls of many of the cells. (**c**) the same cell as 3**a** and 3**b** photographed after 4 min. The blue colour has now become more intense within the formerly yellow compartment. (**d**) a yellow cell stained with 10 % sodium carbonate solution; observe that the intense yellow colour is stronger than in the untreated yellow cell shown in figure 3**a**. (**e**) an individual yellow cell. Some of the contents appear to have been released during sectioning. (**f**) the same cell as 3**e** stained with Nile red and visualized under green light excitation showing intense red fluorescence within the yellow compartment indicating the presence of lipid material. (bs: blue staining; od: oil drop; fod: fluorescent oil drop; scw: stained cell wall; uc: unpigmented cell; yc: yellow cell.) Bar = 50 μm.

cation of the colour makes counting easier although the number of pigmented cells does not change.

Spectra of individual pigmented cells determined microspectrophotometrically before and after the addition of sodium carbonate clearly show the shift in absorption (see Fig. 1*a*, *b*). This could be due to the presence of curcumin derivatives (Harvey, 1981; Tonnensen & Karlsen, 1983), and/or flavonoid-like compounds since the addition of 10 % (w/v) sodium carbonate solution to pure [6]-gingerol does not produce any shift in the absorbance spectrum of this compound with the maximum remaining at 282 nm. When the extracted flavonoids from flowers of four different species were exposed to sodium carbonate a similar shift was observed in absorbance to that recorded by microspectrophotometry for the ginger rhizome cells (data not shown).

Cell counting of cryo-sections under the micro-

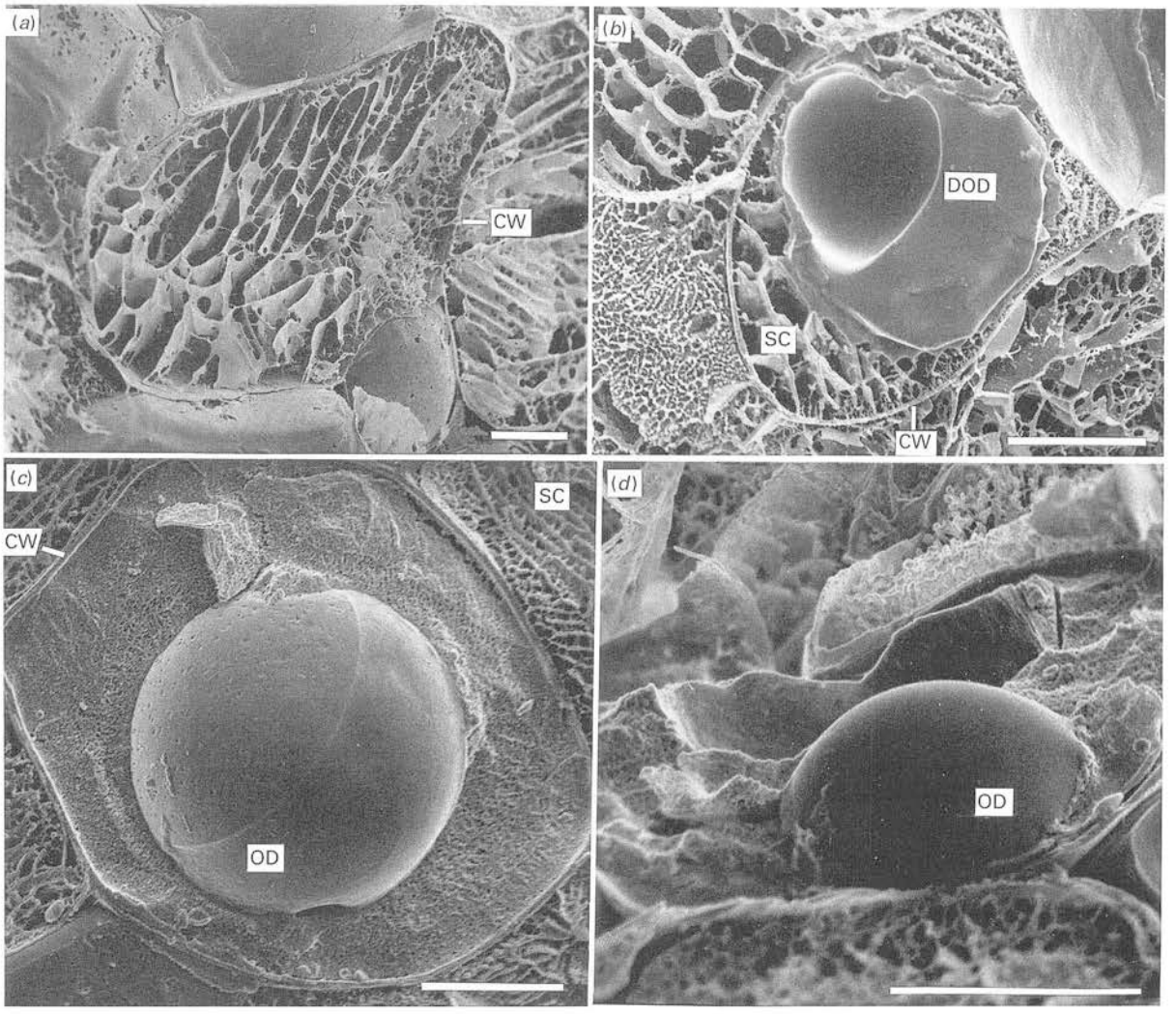

Figure 4. LTSEM micrographs from a cryo-fractured and etched ginger rhizome. (**a**) cell lacking oil with etched cytoplasm showing segregation zones. (**b**) cell with a developing oil drop and partly segregated cytosol. (**c**) and (**d**) two different cells showing oil drops. Note the absence of etched cytoplasm due to their low water content and the segregated cytosol in the surrounding cells. (CW: cell wall; DOD: developing oil drop; OD: oil drop; SC: segregated cytosol.) Bar = 20 μm.

scope revealed that the number of pigmented cells was higher in tissue from mature rhizomes than in micropropagated rhizomes or in adventitious roots. Similar cell types were observed within a population of suspended cells in culture but no estimate of pigmented cell number was made. In Figure 2 the relationship between number of pigmented cells in 30 uniform cryo-sections, after the addition of sodium carbonate, and the concentration of gingerol, the main pungent principle of ginger, is presented. From these data it would appear that there is a reasonable correlation (regression coefficient $r = 0{\cdot}782$) between the two parameters suggesting that the site of gingerol accumulation in the mature rhizome, immature rhizome and adventitious roots lies in the pigmented cells. It was also observed that the intensity of the yellow colour varies between cells of the same cryo-section which may suggest different stages in its formation. It is also probable that the amount of [6]-gingerol will vary between cells perhaps according to size; therefore, a higher regression coefficient would be expected if the cells were uniform. From this it would appear that the yellow cells from all three sources, and also probably those present in suspension cultures, are the repositories for a number of secondary compounds, including the flavonoids, curcumins and gingerol. It is also likely that the yellow cells contain lipid material as the phenolic compounds that constitute the pungent fraction in ginger are toxic in the free state and therefore must be present either dissolved in lipid or as conjugates. It can be seen from Figure 3*a*, *b* and *c* that when a mixture of 1 % (w/v) ferric chloride, 0·5 % (w/v) potassium ferricyanide in water was added to the section the contents of the yellow cells stained intensely blue. Figure 3*a* shows a yellow pigmented cell before the addition of the reagent mixture surrounded by cells with no yellow

content. It can be observed in Figure 3*b* that when, *c.* 30 s after the phenolic reagent mixture was added, a blue colour starts to develop, mainly in the yellow compartment, some of the cell walls also begin to stain blue. After approximately 4 min (see Fig. 3*c*) the blue colour has developed very strongly. At this stage the cell walls have also reacted positively indicating the presence of phenolic compounds within this cell compartment. However, only the contents of yellow cells produce a blue colour with this reagent. These results show that phenols and the flavonoids are located within the same cells. It is also suggestive, bearing in mind the correlation between gingerol content and number of yellow cells, that gingerol is also present (see Fig. 2).

Free phenolic compounds are toxic to plant cells (Brown, 1981) and are stored as conjugates (Harborne, 1984; Sukrasno & Yeoman, 1993) in the vacuoles (Matile, 1990) in the cell wall or in plastids (Wierman, 1981) or in differentiated structures such as oil droplets (Suzuki, Fujiwake & Iwai, 1980). It is possible that gingerol, which is non-polar, is stored in lipids. It has been suggested by Suzuki *et al.* (1980) that capsaicinoids, the phenolic pungent compounds of the chilli pepper, are contained in oil droplets in the placental cells of the maturing fruit. In Figure 3*e* and *f* lipidic material in the cells has been visualized with Nile red dye. The intense red fluorescence is observed only in cells with yellow contents which also react positively for phenolic compounds. This result shows that the flavonoids, phenolics (including gingerol), curcumin derivatives and the essential oils, characteristic of the spice, are accumulated in the same cell type which conflicts with the suggestion of Mangalakumari *et al.* (1984) that there are two cell types in the ginger rhizome, one containing the gingerol and another containing the essential oils. Further evidence for the presence of stored lipid material in these specialized oil cells described by Winton & Winton (1939) may be provided using the freeze etching technique which allows differentiation of organelles and membranes (Robards, 1991). The characteristic appearance of cells without oil drops can be seen in Figure 4*a*; here there is a clear segregation of the cytosol into zones. Cells with a developing oil drop still show a segregated cytosol (Fig. 4*b*). In contrast, cells with a high oil content (Fig. 4*c* and *d*) do not develop an etched cytosol, because of their low water content, and contain a very conspicuous drop of oil. Also it can be seen in Figure 4*b* and *c* that the surrounding cells with no oil content show segregation of their cytosol. In conclusion, all the results presented here indicate that the accumulation of the gingerol occurs within cells with yellow contents. These cells also contain the essential oil of the spice suggesting the presence of only one cell type as a storage compartment for both the phenolic pungent principles and the essential oils.

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